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Studies on the toxicity of 6-hydroxydopa and hydrogen peroxide in the central nervous system

Evans, Jacqueline Meryl, Ph.D.

City University of New York, 1990

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STUDIES ON THE TOXICITY OF 6-HYDROXYDOPA AND HYDROGEN PEROXIDE IN THE CENTRAL NERVOUS SYSTEM

by

JACQUELINE M. EVANS

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fufillment of the requirements for the degree Doctor of Philosophy, The City University of New York.

1990

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree Doctor of Philosophy.

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Abstract

Studies on the Toxicity of 6-Hydroxydopa
and Hydrogen Peroxide in the Central Nervous System

by

Jacqueline M. Evans

Adviser: Professor Gerald Cohen

The effects of hydrogen peroxide production on central catecholamine neurons were explored using two independent means of peroxide production. In the first set of experiments the peroxide-generating catecholamine neurotoxin 6-hydroxydopa (6-OH-DOPA, 2,4,5-trihydroxyphenylalanine) was studied. 6-OH-DOPA destroys central and peripheral noradrenergic neurons while sparing dopaminergic neurons. Previous studies indicated that 6-OH-DOPA toxicity is mediated by the formation of 6-hydroxydopamine (6-OHDA, 2,4,5-trihydroxyphenylethylamine).

The levels of 6-OH-DOPA and 6-OHDA in mouse brain were measured after systemic administration of 6-OH-DOPA. Levels of 6-OHDA in mouse striatum were remarkably stable. Experiments with reserpine indicated that the stability of 6-OHDA was largely dependent upon storage in synaptic vesicles. 6-OHDA levels were increased after inhibition of monoamine oxidase, inhibition of peripheral decarboxylase, or after inhibition of axonal uptake. These data are in accord with the strong localization of the decarboxylase to catecholamine terminals and suggest that 6-OH-DOPA is converted to 6-OHDA within catecholamine nerve terminals.

Administration of a neurotoxic dose of 6-OH-DOPA caused a near total reduction in both cortical norepinephrine levels and synaptosomal uptake of ^{3}H-norepinephrine, whereas striatal dopamine levels and uptake of ^{3}H-dopamine remained unchanged. At the same time, 6-OHDA levels were 8.8-fold higher in the striatum (5.54 ug/g) than in the frontal cortex (0.63 ug/g). These data showed that striatal dopamine neurons were resistant to 6-OH-DOPA-induced toxicity despite the presence of sizable amounts of 6-OHDA.

In a second set of experiments mice were treated with reserpine in order to enhance peroxide production by monoamine oxidase. Reserpinization caused a prolonged decrease in striatal dopamine levels; however, synaptosomal uptake of ^{3}H-dopamine and ^{3}H-mazindol binding were unaltered. A significant (10.9 %) decrease in striatal tyrosine hydroxylase activity at one week after reserpine may however partially explain the long-lasting decrease in dopamine levels. Additional data provide evidence for a possible role of monoamine oxidase in the return of striatal dopamine levels after reserpine.

Acknowledgements

To Thelma Gorfien, who first sparked my quest for knowledge in the world of living things; and to my parents, Donald and Lorraine Evans, who continue to be a never-ending source of love and support.

The following publications have resulted from the work described in this dissertation prior to its submission to the City University of New York.

Evans J. M. and Cohen G. (1989) Studies on the formation of 6-Hydroxydopamine in Mouse Brain After Administration of 2,4,5-Trihydroxyphenylalanine (6-Hydroxydopa). J. Neurochem. 52, 1461-1467.

Evans J. M. and Cohen G. (1989) Can Trace Amounts of neurotoxins destroy dopamine neurons? Neurochem. Int. 15, 127-129.

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Dr. Majorie B. Lees
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Re: MS 88-252

July 17, 1989

Dear Dr. Lees:

I am writing to request a copyright release for a manuscript entitled "Studies on the formation of 6-hydroxydopamine in mouse brain after administration of 2,4,5-trihydroxyphenylalanine (6-hydroxyDOPA) by Jacqueline M. Evans and Gerald Cohen which appeared in the Journal of Neurochemistry (1989) Vol. 52, 1461-1467. The material will appear in my doctoral dissertation submitted to the City University of New York, in partial fulfillment of the requirements for the degree Doctor of Philosophy.

I thank you in advance for assistance.

Sincerely,

Jacqueline M. Evans

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List of abbreviations

diethylenetriaminepenta-acetic acid (DTPA)

diethylmaleate (DEM)

3,4-dihydroxybenzylamine (DHB)

3,4-dihydroxyphenylacetic acid (DOPAC)

L-3,4-dihydroxyphenylalanine (L-DOPA)

dopamine (3,4-dihydroxyphenylethylamine, DA)

5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)

glutathione (reduced form, GSH; oxidized form, GSSG)

6-hydroxydopa (2,4,5-trihydroxyphenylalanine, 6-OH-DOPA)

6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine, 6-OHDA)

high performance liquid chromatography (HPLC)

homovanillic acid (HVA)

hydrogen peroxide (H_2O_2)

intracerebroventricular (i.c.v.)

intraperitoneal (i.p.)

intravenous (i.v.)

metaraminol (MA)

monoamine oxidase (MAO)

beta-nicotinamide-adenine dinucleotide phosphate (reduced form, NADPH)

norepinephrine (1-(3,4-dihydroxyphenyl)-2 aminoethanol, NE)

perchloric acid (PCA, $HClO_4$)

subcutaneous (s.c.)

Chapter 1: Overview

1.1 Introduction

In understanding any pathological process it is necessary to localize these changes to a discrete region and/or cell type. This type of analysis has been fundamental to the understanding of neurodegenerative disease. For example, the major hallmark of Parkinson's disease is a loss of the melanized cells of the pars compacta of the substantia nigra (Ehringer and Hornykiewicz, 1960; Bernheimer et al., 1973), and Alzheimer's disease is characterized by a loss of cells in the nucleus basalis of Meynert (Davies and Verth, 1976), while the most prominent finding in Huntington's chorea is a loss of striatal neurons (Bernheimer et al., 1973; Beal et al., 1986).

In the case of Parkinson's disease, it has been suggested that the neurotransmitter involved, dopamine (DA), plays a key role in the progression of this disease (Cohen, 1986). Hornykiewicz (1983) has described a compensatory increase in "presynaptic activity" in the remaining neurons of the nigrostriatal system. Such changes are characterized by an increase in DA turnover as seen by an increase in the ratio of the metabolite, homovanillic acid (HVA) to parent amine, DA (Hornykiewicz, 1979). More recently, evidence was presented that HVA rises as DA falls in the putamen during aging in human subjects (Hornykiewicz, 1988).

It is of interest that these compensatory changes need

not be considered as being secondary only to a considerable insult such as Parkinson's disease, in which at least 80% of the dopaminergic neurons of the nigra must be lost before even mild clinical symptoms appear. In fact, these same types of changes (i.e., presynaptic overactivity of dopaminergic neurons) take place during normal aging. In a postmortem study of "young" versus "old" healthy controls, the levels of DA in the caudate nuclei of older individuals (as measured as percent of "young" controls), decreased to a greater degree than did the levels of HVA, resulting in an increase in the ratio of HVA to DA (Hornykiewicz, 1983).

Therefore increases in DA turnover can be viewed as being characteristic of normal senescence as well as neurodegenerative disease. Moreover, current evidence indicates that increases in hydrogen peroxide (H_2O_2), produced through the metabolism of DA by monoamine oxidase, may lead to oxidative and thus damaging events. In a recent study, Spina and Cohen (1989) measured sizable increases in glutathione disulfide (oxidized glutathione, GSSG) after reserpine-induced increases in DA turnover. This effect was even greater when the DA receptor blocker, haloperidol, was used in place of reserpine (Cohen and Spina, 1989). While an accumulation of H_2O_2 can lead to oxidative events such as lipid peroxidation, elevations in GSSG could lead to the inactivation of sulfhydryl-dependent enzymes through the formation of mixed disulfides.

It is conceivable that small increases in presynaptic activity that occur over the course of normal aging might be associated with a subtle form of oxidant stress that need not be associated with overt destruction, yet might be related to a metabolic impairment. As reviewed by Morgan and Finch (1988), there is a large literature describing age-related decreases in DA levels in the striatum of humans, primates, rats, and mice. It is not clear, however, that decreases in DA levels are associated with overt loss of cells. DA levels are reduced by 20-25% in the brains of aged (30 months) C57BL/6J mice (Finch, 1973; Osterberg et al., 1981). McNeill and coworkers (1988) counted immunocytochemically stained cells in the substantia nigra and striatum of C57BL/6 mice (3, 6, 10, 20, 25, and 30 months). Although there were 13% less tyrosine hydroxylase stained cells in the substantia nigra of aged mice (30 months), this change was not statistically significant. The authors conclude that age related decreases in striatal DA levels are not associated with a loss of dopaminergic neurons.

In a separate study McNeill, Koek, and Haber (1984) observed that age related decreases in histofluorescence for DA were detectable in the substantia nigra of C57BL/6NNia mice by 10 months of age (mature adult). Decreases in histofluorescence progressed steadily between 10 months and 30 months (senescent adult) and were associated with an increase in the number of enlarged, fluorescent, axonal

dilations in dopaminergic fibers, as well as a progressive accumulation of lipofuscin pigment in dopaminergic cell bodies. In contrast, the number of tyrosine hydroxylase positive cells appeared similar across age groups and did not decrease with age. Taken together these observations seem to suggest that marked decreases in DA histofluorescence and alterations in the morphology of dopaminergic neurons do not indicate a loss of cells, but rather some type of age related metabolic change.

In regard to the data on human brain, what seems clear, is that DA levels decrease in aged human brain; however, what is not clear is whether or not an overt cell loss is associated with the decline in DA levels. McGeer et al. (1977) studied 13 human brains and reported a 50% decline in nigral cell bodies (cresyl violet stain) between the ages of 10 and 80 years (7% decline per decade). However, Mann and Yates (1983) and Mann (1984) studied 4 brains at 15 years of age, 13 brains at 65 years, and 13 at 84 years, and found only a 7% fall in cell count (hematoxylin-eosin stain) between 15-65 years (1.4% decline per decade), and a 21% drop between 65-84 years (11% decline per decade). More recently, McNeill et al (1988) counted tyrosine hydroxylase-positive cells in human substantia nigra and reported no decrease in dopaminergic neurons over the age group studied (43-89 years).

Therefore the possibility exists that age related

increases in DA turnover bear some relationship to decreases in DA levels and alterations in the morphology of dopaminergic neurons. Because elevated DA turnover is associated with a concomitant increase in peroxide production, it is of interest study the effects of H_2O_2 on dopaminergic neurons. The studies that follow explore the effects of peroxide on dopaminergic neurons via two independent routes.

The first set of experiments (Chapters 3-5) involves the catecholamine neurotoxin 6-hydroxydopa (6-OH-DOPA). Although the mechanism of action of 6-OH-DOPA is known to involve the formation of 6-hydroxydopamine (6-OHDA), the effects of central or peripheral administration of 6-OH-DOPA differ from those of local stereotaxic injection of 6-OHDA. While 6-OHDA is toxic to dopaminergic and noradrenergic neurons, 6-OH-DOPA is selectively toxic to central noradrenergic neurons and spares central dopaminergic neurons (as reviewed by Jacobowitz, 1973 and Kostrzewa and Jacobowitz, 1974). This observation is of interest in the current study because the mechanism of toxicity of 6-OHDA involves the formation of H_2O_2 (Heikkila and Cohen, 1972, Cohen et al., 1976; Graham et al., 1978). By studying the resistance of dopaminergic neurons to a peroxide-generating neurotoxin, it may be possible to gain a better understanding of the changes in these same neurons during periods of increased DA turnover.

The second set of experiments (Chapter 6) explores the

effects of H_2O_2 produced through reserpine-induced increases in DA turnover. Reserpine binds irreversibly to amine storage vesicles causing rapid and long-lasting depletions of central and peripheral monoamines (Carlsson et al., 1957). In addition, the effects of reserpinization may contain components that also occur during normal aging (lowered DA levels and increased DA turnover in intact dopaminergic neurons). Moreover, as mentioned earlier, reserpine-induced increases in DA turnover have been associated with an oxidant stress (Spina and Cohen, 1989). The current study explores the possibility that increases in DA turnover may influence the rate of reappearance of DA levels after reserpinization. The effects of reserpine may reflect on changes take place during normal aging.

1.2 Background: 6-Hydroxydopa

1.2.1 6-Hydroxydopa

6-hydroxydopamine (2,4,5-trihydroxyphenylalanine, 6-OHDA) is well known for its neurotoxic effects on both dopaminergic and noradrenergic neurons (as reviewed by Kostrzewa and Jacobowitz, 1974). Its amino acid precursor, 6-hydroxydopa (2,4,5-trihydroxyphenylalanine, 6-OH-DOPA) has also been well studied (see reviews by Jacobowitz, 1973 and Kostrzewa and Jacobowitz, 1974). Although 6-OH-DOPA is readily decarboxylated to 6-OHDA (Ong et al., 1969), the neurotoxic effects of 6-OH-DOPA differ from those of 6-OHDA

in that 6-OH-DOPA-induced toxicity is selective for central and peripheral noradrenergic neurons (Jacobowitz, 1973; Kostrzewa and Jacobowitz, 1974). The reason for the relative resistance of dopaminergic neurons to 6-OH-DOPA remains to be elucidated.

The following sections contain a review of what is currently known regarding the mechanism of action and neurotoxic effects of 6-OH-DOPA.

1.2.2 Synthesis and biological activity

Ong et al. (1969) were among the first groups to describe the synthesis and toxic effects of 6-OH-DOPA. Using purified enzyme, these workers demonstrated the decarboxylation in vitro of 6-OH-DOPA to 6-OHDA by the L-aromatic amino acid decarboxylase (Ong et al., 1969). In addition, these workers were able to demonstrate that decarboxylation was necessary for toxicity; they showed that the depleting effects on cardiac norepinephrine (NE) could be blocked with the peripheral decarboxylase inhibitor, RO4-4602 (N-DL-seryl-N'-2,3,4-trihydroxybenzylhydrazine, 50 mg/kg i.p., 30 minutes) (Ong et al., 1969). In contrast, pretreatment with the same compound (RO4-4602) enhanced the depleting effects of 6-OH-DOPA on whole brain NE levels, presumably by blocking the wasteful decarboxylation of 6-OH-DOPA in the periphery (i.e., by the liver or by cerebral capillaries), and thus allowing for a higher concentration

of 6-OH-DOPA to be present in brain.

Using a dose of RO4-4602 large enough to inhibit both central and peripheral decarboxylation of 6-OH-DOPA (500 mg/kg i.p., 30 minutes), Sachs and Jonsson (1972a) blocked decreases in ^{3}H-NE uptake in both atria and cerebral cortex slices after 6-OH-DOPA. These data indicate that decarboxylation is a requirement for toxicity in the CNS as well as in the periphery.

The synthesis and biological activities of the optical isomers of 6-OH-DOPA were reported by Berkowitz et al. in 1971. In this study, the (-)-isomer was found to be more potent than the (+)-isomer in depleting central and peripheral stores of NE. In the periphery, the heart was found to be more sensitive than the adrenals to the depleting effects of both isomers. A low dose of (-)-6-OH-DOPA (25 mg/kg i.p., given twice at 24 hour intervals) decreased cardiac NE by greater than 90%, while the same dose of the (+)-isomer decreased cardiac NE by only about 50%. NE levels in the adrenals were reduced by 50% by a larger dose of (-)-6-OH-DOPA (100 mg/kg i.p., given twice at 24 hour intervals) and by 20% by the same dose of the (+)-isomer. In contrast, brainstem levels of NE were decreased by 34% by the larger dose of (-)-6-OH-DOPA, and were unaffected by the same dose of the (+)-isomer. The lack of affect of (+)-6-OH-DOPA on central noradrenergic neurons may be due to relatively lesser transport of the (+)-isomer across the blood-brain barrier.

1.2.3 Pharmacological Interactions

1.2.3.1 Inhibition of decarboxylation

6-OH-DOPA has been given in conjunction with several different peripherally-acting decarboxylase inhibitors with variable effects on central and peripheral toxicity. Carbidopa (MK-486, L-alpha-hydrazine methyldopa) provided almost complete protection against decreases in cardiac NE (Kostrzewa and Jacobowitz, 1972), while at the same time augmenting the depletion in brain NE (Kostrzewa and Jacobowitz, 1973). Similar results were obtained by Ong et al. (1969) who used a low dose of RO4-4602 (50 mg/kg i.p., 30 minutes) in order to block peripheral decarboxylation of 6-OH-DOPA, while a much larger dose of this drug (500 mg/kg i.p., 30 minutes) simultaneously inhibited decarboxylase activity and blocked 6-OH-DOPA-induced toxicity in the periphery and the CNS (Sachs and Jonsson, 1972a). In contrast, pretreatment with the peripheral decarboxylase inhibitor MK-485 (DL-(3,4-dihydroxyphenyl)-alpha-hydrazino-alpha-methyl-propionic acid) partially blocked 6-OH-DOPA induced decreases in both cardiac NE (Corrodi et al., 1971) and ^{3}H-NE uptake into atria (Sachs and Jonsson, 1972a), but did not augment the effects of 6-OH-DOPA on whole brain levels of NE (Corrodi et al., 1971) or ^{3}H-NE uptake into slices of cerebral cortex (Sachs and Jonsson, 1972a). Similarly, the peripheral decarboxylase inhibitor NSD-1055

(4-bromobenzyloxyamine phosphate) partially blocked decreases in cardiac NE (Kostrzewa and Jacobowitz, 1972), but did not enhance decreases in brain NE after 6-OH-DOPA (Kostrzewa and Jacobowitz, 1973). The absence of a potentiating effect of either MK-485 or NSD-1055 on the toxicity of 6-OH-DOPA to central noradrenergic neurons may be due to a relatively weaker effect of these drugs on blocking peripheral decarboxylation as suggested by incomplete protection of peripheral toxicity. On the other hand, Jacobowitz (1973) has suggested that, the inability of MK-485 to potentiate the toxic effects of 6-OH-DOPA on central noradrenergic neurons (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson, 1972a; Kostrzewa and Jacobowitz, 1973) may be due to the appearance of enlarged, swollen, nonterminal axons which would tend to accumulate neurotransmitter and thus obscure decreases in NE levels in terminals.

1.2.3.2 Inhibition of monoamine oxidase

The toxic effects of 6-OH-DOPA can be enhanced by the inhibition of monoamine oxidase (MAO); this is consistent with previou· a study by Jonsson and Sachs (1971) which characterized 6-OHDA as a substrate for MAO. The toxic effects of 6-OH-DOPA are also affected by route of administration (i.e., i.p. vs. i.v.; Kostrzewa and Jacobowitz 1972, 1973) Pretreatment with nialamide (100

mg/kg i.p.) 2 hours prior to 6-OH-DOPA (100 mg/kg i.p.) led to lasting decreases in the uptake of ^{3}H-amines (NE and metaraminol, MA) and the density of nerve terminals as revealed by catecholamine histofluorescence in the periphery and in the CNS. When given alone at this dose, 6-OH-DOPA failed to significantly alter either of these parameters (Sachs and Jonsson, 1972a,b).

Similarly, pretreatment with tranylcypromine (5 mg/kg i.p.) one hour prior to 6-OH-DOPA (100 mg/kg i.v.), lowered levels of NE in heart (ventricle) and whole brain. These decreases did not however reach statistical significance when compared to animals receiving 6-OH-DOPA alone (Kostrzewa and Jacobowitz, 1972, 1973). In addition, pargyline (100 mg/kg s.c.), has also been used in conjunction with 6-OH-DOPA (80 mg/kg i.v.) in order to potentiate 6-OH-DOPA induced decreases in brainstem NE and epinephrine (VonVoigtlander and Losey, 1978). These data suggest that MAO inhibitors act by blocking the metabolism of 6-OHDA by MAO. In addition, these results emphasize that the mechanism of toxicity of 6-OH-DOPA involves its conversion to 6-OHDA.

1.2.3.3 Inhibition of catechol-O-methyltransferase

In contrast to its interaction with MAO, 6-OHDA does not appear to be a substrate for the enzyme catechol-O-methyltransferase, as pretreatment with an inhibitor of this enzyme, beta-isopropyltropolone (thujaplicin) (400 mg/kg

i.p.) did not enhance or prevent the toxic effects of 6-OH-DOPA on central or peripheral adrenergic neurons (Kostrzewa and Jacobowitz, 1972,1973).

1.2.4 Specificity of neurotoxic effects

6-OH-DOPA is selectively toxic to central noradrenergic neurons, whereas central dopaminergic terminals are resistant (Corrodi et al., 1971; Jacobowitz and Kostrzewa 1971; Clarke et al., 1972; Sachs and Jonsson 1972a,b; Kostrzewa and Jacobowitz, 1973; Richardson and Jacobowitz, 1973; McClean et al. 1980, Kantak et al., 1981; and Cornwell-Jones and Bollers, 1983). The specificity of 6-OH-DOPA for central noradrenergic neurons differs from the known effects of intracranial administration of 6-OHDA. Stereotaxic administration (i.c.v.) of 6-OHDA (100-500 ug) is a commonly used means of destroying both dopaminergic and noradrenergic neurons (Iversen and Uretsky, 1971; see also review by Kostrzewa and Jacobowitz 1974). However, it is possible to selectively destroy central noradrenergic neurons with small amounts (25-75 ug) of 6-OHDA (Breese and Traylor, 1971; Iversen and Uretsky, 1971). This effect seems to parallel the selective toxicity of 6-OH-DOPA for central noradrenergic neurons.

1.2.4.1 Effects of systemic administration

1.2.4.1.1 Neurochemical findings

Kostrzewa and Jacobowitz (1973) studied the acute affects of intravenous 6-OH-DOPA on whole brain levels of NE and DA in mice. Low doses of 6-OH-DOPA (20 and 50 mg/kg i.v.) reduced whole brain levels of NE by approximately 30 percent at 3 hours, whereas higher doses (100 and 150 mg/kg i.v.) decreased NE levels by 70 to 75 percent. At 24 hours, NE remained significantly reduced by all but the lowest dose of 6-OH-DOPA; higher doses of 6-OH-DOPA decreased NE levels by 15-53 percent.

In contrast to the data on noradrenergic sensitivity to 6-OH-DOPA, evaluation of the effects of 6-OH-DOPA on dopaminergic neurons indicates that these neurons are relatively resistant. Only a single study, by Kostrzewa and Jacobowitz (1973), reports a decrease in DA levels after administration of a relatively high dose of 6-OH-DOPA by the i.v. route. This decrease was small and transient as compared to decreases in NE levels, which were much larger and longer-lasting. In the latter study, whole brain levels of DA were reduced by 30% at 3 hours after 6-OH-DOPA (150 mg/kg i.v.), whereas the same dose reduced whole brain NE levels by 75%. In addition, DA levels had fully recovered by 5 days after 6-OH-DOPA, whereas decreases in whole brain NE persisted for 66 days (Jacobowitz and Kostrzewa, 1971).

Moreover, other investigators report a complete resistance of dopaminergic terminals to 6-OH-DOPA, even at

much larger doses. For example, whole brain NE was reduced by 80% while DA levels were unchanged at 3 and 4 hours after 400 mg/kg (i.v.) of 6-OH-DOPA (Clarke et al., 1972).

Sachs and Jonsson (1972a,b) studied the effects of 6-OH-DOPA administration (3 x 100 mg/kg i.p. injections at 24 hour intervals, with nialamide pretreatment, 100 mg/kg i.p., 2 hours) on ^{3}H-MA uptake in slices of cerebral cortex and striatum at 16 hours, as well as at 7, 14, 28, and 42 days after the last 6-OH-DOPA injection. ^{3}H-MA uptake was reduced by 65% at 16 hours after the last 6-OH-DOPA injection and had not recovered by 42 days. Decreases in whole brain levels of NE were monitored simultaneously for all but the 42 day time point and paralleled the observations with cortical slices. In agreement with the previous data regarding a lack of effect of 6-OH-DOPA on DA levels, uptake of ^{3}H-MA into striatal slices was unchanged at all time points studied. In addition, uptake of ^{3}H-DA at one week after the last 6-OH-DOPA injection was unaffected.

1.2.4.1.2 Histochemical findings

Neurochemical findings were confirmed at the light microscopic level by catecholamine histofluorescence (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson 1972a,b; Jacobowitz, 1973; Richardson and Jacobowitz, 1973). Jacobowitz and Kostrzewa (1971) examined the brains of mice at various times after 6-OH-DOPA administration (100 and 150 mg/kg i.v.). At 3 hours after 6-OH-DOPA there was a

decrease in terminal varicosities in areas known to receive noradrenergic innervation as well as the appearance of a small number of intensely fluorescent preterminal axons. After 24 hours, the number of preterminal smooth axons had greatly increased. The preterminal processes were larger and smoother than terminal varicosities, and were not contained within in a plexus of arborizing fibers. This increased fluorescence was thought to be due to a proximal accumulation of NE within noradrenergic neurons similar to that associated with axotomy, 6-OHDA-induced degeneration, or stereotaxic lesions.

Upon examination, noradrenergic cell bodies in the brain stem appeared similar to those of control animals. Preterminal processes were still visible at 7 days after 6-OH-DOPA; however, by 2 weeks, areas receiving noradrenergic innervation (i.e., the hypothalamus, preoptic area, and septum, as well as the dorsal motor nucleus of the vagus nerve, the trigeminal mesencephalic nucleus, and the nucleus interstitialis stria terminalis) appeared similar to those of control animals, with the exception of the cerebral cortex, cerebellum, and hippocampus which continued to show decreased fluorescence.

Areas receiving dopaminergic innervation or containing dopaminergic cell bodies (i.e., substantia nigra, striatum, nucleus accumbens, dorsal part of the nucleus interstitialis stria terminalis, and olfactory bulb) did not show any

apparent signs of histochemical alterations.

These findings were confirmed by Sachs and Jonsson (1972a,b). Fluorescence histochemical observation of smears of cerebral and cerebellar cortex revealed a decreased number of noradrenergic nerve terminals at both 16 hours and 4 weeks after 6-OH-DOPA administration. At 16 hours after 6-OH-DOPA, there were large number of strongly fluorescent fibers; the number of these fibers had decreased somewhat by 4 weeks. Histofluorescence of noradrenergic cell bodies was not noticeably altered.

In agreement with Jacobowitz and Kostrzewa (1971) fluorescence histochemistry of DA-containing areas was not affected by 6-OH-DOPA administration (Sachs and Jonsson, 1972a,b). In the hypothalamus, the heavy innervation of the external layer of the median eminence by dopaminergic terminals showed unaltered histofluorescence. Similarly, the fluorescence morphology of both the dopaminergic terminals of the striatum as well as their cell bodies in the substantia nigra remained identical to that of control animals.

Therefore the histological observations described above are in agreement with the neurochemical data and clearly demonstrate that dopaminergic neurons differ from noradrenergic neurons in their resistance to systemically administered 6-OH-DOPA.

1.2.4.2 Effects of central administration

The studies discussed in Section 1.4.1 review the

neurochemical and histological reports on the neurotoxic effects of systemically administered 6-OH-DOPA. These data indicate that 6-OH-DOPA given via subcutaneous, intraperitoneal, or intravenous routes is selectively toxic to central noradrenergic neurons. In addition, central dopaminergic neurons remain resistant even when 6-OH-DOPA is administered in conjunction with peripherally acting decarboxylase inhibitors, MAO inhibitors, or when given alone at high doses. The material that follows reviews the effects of central administration of 6-OH-DOPA.

1.2.4.2.1 Neurochemical findings

Richardson and Jacobowitz (1973) studied the neurotoxic effects in rats of intracerebroventricular (i.c.v.) administration of 6-OH-DOPA (45, 60, 90, 135, or 180 ug). Two days after 6-OH-DOPA, NE levels were significantly reduced in the telencephalon and diencephalon at all doses tested. Hindbrain NE was reduced by the 60, 90, 135, and 180 ug doses, and cerebellar NE was reduced by 90, 135, and 180 ug doses. DA levels in the telencephalon were unaffected by all doses.

Regional NE levels were also assessed at 2, 4, 7, 14, and 70 days after the 90 ug dose of 6-OH-DOPA. At 2 days, levels of NE in the telencephalon, diencephalon, hindbrain, and cerebellum were reduced by 40%-45%. Telencephalic NE was still reduced by 40% at 14 days after injection and had

returned to normal at 70 days, whereas NE in the diencephalon continued to fall over the first 14 days and was still reduced by 33% at 70 days after injection. Hindbrain NE was maximally reduced at 2 days and gradually returned to control values by 70 days after injection, while NE in the cerebellum continued to decrease over the first 14 days was still reduced by 70 days after injection.

In addition, NE levels in cervical-thoracic spinal cord were reduced by 35% at 2 days and 67% at 14 days after injection of 6-OH-DOPA (90 ug), while NE in the heart (ventricles) was unaffected at any of the time points tested. DA levels in the telencephalon were unaltered at any of the time points tested after injection of 6-OH-DOPA (90 ug).

1.2.4.2.2 Histochemical findings

The results of histological studies by catecholamine histofluorescence after i.c.v. 6-OH-DOPA were similar to those of peripheral administration. Histological studies were carried out at 1, 2, 4, 7, 14, and 70 days after injection of 6-OH-DOPA (90 ug). At 1 and 2 days there was a marked appearance of many intensely fluorescent axons with swollen and distorted segments. Fluorescent fibers were located in the medulla oblongata, reticular formation, proximal to the locus ceruleus, cerebellar peduncle, habenula, preoptic area, stria medullaris, septal area, cingulum, and the dorsomedial region of the striatum.

There was also a prominent reduction in the number of noradrenergic nerve terminals (i.e., in the hypothalamus, internal layer of the median eminence, preoptic area, septum, hippocampus, habenula, cortex, and cerebellum) that was most noticeable near the ventricular region (i.e., the periventricular, paraventricular, and dorsomedial nuclei but not the lateral nucleus of the hypothalamus). Similarly, there was a greater decrease in fibers in the preoptic periventricular and the preoptic medial nuclei than in the lateral preoptic nucleus or the median forebrain bundle.

In contrast, areas receiving dopaminergic innervation (i.e., striatum, olfactory bulb, nucleus accumbens, and dorsal part of nucleus interstitialis stria terminalis) appeared normal. The noradrenergic, dopaminergic, and serotonergic cell bodies in the mesencephalon and medulla were also unaltered.

At 4 to 7 days after injection there was still a considerable amount of swollen fluorescent processes; however, small axonal sprouts were also noted budding off main trunks. Sprouting was more noticeable at 7 than at 4 days at which point there was still a marked loss of terminals. Analysis at 14 days revealed increased sprouting (most noticeable in the hypothalamus, preoptic area, median forebrain bundle, septum, and medulla), and the continued presence of swollen fluorescent axons. By 70 days the regions of greatest regeneration received a near normal

noradrenergic innervation but continued to contain more intensely fluorescent fibers. Areas containing monoamine cell bodies appeared normal at 4, 7, 14, and 70 days after injection.

In conclusion, the results of neurochemical and histochemical analysis of the brains of rats after central (i.c.v.) administration of 6-OH-DOPA were essentially the same as after peripheral (s.c., i.p., or i.v.) administration of 6-OH-DOPA and indicate that the resistance of dopaminergic terminals to 6-OH-DOPA is not a function of route of administration.

1.2.5 Studies with fetal or neonatal animals

In contrast to studies involving 6-OHDA-treated mature animals in which 6-OHDA must be injected directly into the brain, frequently as a large bolus, studies involving 6-OHDA-treated neonates do not require central administration because 6-OHDA readily crosses the immature blood-brain barrier. Therefore studies on 6-OHDA-treated neonates are more easily compared to experiments with 6-OH-DOPA-treated animals.

6-OHDA treatment of neonates is also selective for noradrenergic neurons. Tassin et al. (1975) studied the effects of 6-OHDA given to fetal and neonatal rats. 6-OHDA was given i.p. to fetal rats (day 17 of gestation). At 40 days after birth, cerebral cortex and cerebellum were characterized by a lack of noradrenergic innervation,

whereas the dopaminergic innervation of the cerebral cortex was not reduced as indicated by unaltered DA levels, synthesis of ^{3}H-DA from ^{3}H-tyrosine, as well as benztropine-sensitive but desipramine-resistant uptake of ^{3}H-DA.

This study indicates that brain area does not play a role in the resistance of dopaminergic terminals to 6-OHDA-induced toxicity and demonstrates that dopaminergic terminals are selectively resistant even when present to a comparable extent in the same milieu. These data indicate the existence of intrinsic differences between dopaminergic and noradrenergic neurons with regard to their sensitivity to 6-OH-DOPA.

1.3 Background: Reserpine-induced increases in DA turnover

1.3.1 Effects of reserpinization

The effects of reserpine on monoaminergic nerve terminals have been well documented. Reserpine binds irreversibly to amine storage granules and inhibits Mg^{++}-ATP dependent uptake of amines into vesicles (Carlsson, Hillarp, and Waldeck, 1963; Dahlstrom, Fuxe, and Hillarp, 1965). Early studies showed that reserpine causes an immediate reduction in central and peripheral monoamine levels (Carlsson et al., 1957; Dahlstrom and Haggendal, 1966; Haggendal and Dahlstrom, 1971). The time course of reappearance of monoamine levels in brain after reserpinization is still of current interest. The decrease

in monoamine levels is suprisingly long- lasting. Recent work by Ponzio et al. (1984) and Algeri et al. (1987) indicates that at 3 weeks following a single injection of reserpine (5 mg/kg i.p.), levels of monoamines (DA, NE, serotonin, and epinephrine) in striatum, limbic area, hypothalamus, and hippocampus, remained below control values.

Early reports suggested that the return of monoamine levels was dependent on the synthesis of new vesicles as well as the transport of newly synthesized vesicles to the terminal region (Dahlstrom and Haggendal, 1966). However, work by Glowinski and coworkers (1966) indicates that the ability to take up and store neurotransmitter recovers quite early, as compared to the long-lasting reduction in neurotransmitter levels. These workers showed that although uptake and storage of ^{3}H-NE injected (i.c.v) was largely reduced at 6 hours after reserpine (2 mg/kg i.p.), storage recovered dramatically during the next 24-48 hours, and had returned to normal by 8 days after reserpine, at which point NE levels were still reduced by 60%.

Similarly, Anden, Magnusson, and Waldeck (1964) showed that for peripheral sympathetics, nerve function, as measured by stimulation-induced protrusion of the eyeball, while absent between 3 and 30 hours after reserpine (10 mg/kg i.p.), had begun to recover by 48 hours, and was fully recovered by 72 hours after reserpine. Recovery of

functionality seemed to parallel the recovery of the ability to store ^{3}H-NE by the sympathetic innervation of the heart, which had recovered fully by about 6 days after reserpinization, whereas NE levels at this time were less than 40% of controls. Therefore, although resynthesis of vesicles is likely to be important in the return of amine levels, it seems possible that other factors might be involved as well.

In addition to causing a sudden loss of monoamine levels, reserpinization also causes a pronounced increase in the levels of metabolites. In the case of striatal DA, reserpine causes an immediate fall in DA levels that is concomitant with an increase in the metabolites, DOPAC (dihydroxyphenylacetic acid) and HVA. These changes are due to the metabolism of DA by MAO. Current evidence indicates that increased turnover of DA by MAO may have detrimental consequences for the dopaminergic neuron. Spina and Cohen (1989) have demonstrated that at 2 hours following reserpine (10 mg/kg i.p.) levels of oxidized glutathione (GSSG) increase by 87%. This increase in GSSG is due to the detoxification (by glutathione peroxidase) of the H_2O_2 produced by MAO. An accumulation of H_2O_2 can initiate oxidative events such as lipid peroxidation or the oxidation of sulfhydryl groups on proteins. H_2O_2 can oxidize cellular components either directly or through the production of other reactive species such as superoxide (O_2^-) or the hydroxyl radical ($^{\cdot}OH$) (Cohen, 1983).

Similarly, GSSG can inactivate sulfhydryl-dependent enzymes by forming mixed-disulfides (Offerman et al., 1984; Bregelius et al., 1985). Alternatively, GSSG, can cause alterations in enzyme activity by acting as a "third messenger" (Gilbert, 1982). Moreover, a severe reduction in cellular levels of reduced glutathione (GSH) would further sensitize the cell to peroxide-mediated damage by compromising glutathione peroxidase activity.

Therefore reserpine-induced increases in DA turnover are associated with an oxidant stress (elevated H_2O_2 and GSSG) as well as prolonged decreases in DA levels. The concept of accelerated DA metabolism by MAO as a potential "biological stressor" (Cohen, 1986) may be germane to our understanding of certain naturally occuring situations. During periods of increased firing, and hence increased release and reuptake, elevated DA turnover by MAO might set the stage for a "self-generated oxidative stress" (Cohen, 1986).

Hefti et al. (1980) provided empirical evidence that DA turnover increases in rats with incomplete lesions of the substantia nigra. By administering graded doses of 6-OHDA, these workers produced a graded decrease in DA levels. Lesions producing a 66% or greater reduction in DA levels caused an increase in synthesis and release of DA by the remaining dopaminergic neurons as indicated by increased levels of DOPAC and HVA. These data suggest that after a

partial destruction of the nigrostriatal system, the remaining neurons compensate by working harder, as seen by increases in synthesis, release, and metabolism of neurotransmitter.

The experimental paradigm for increased DA turnover described by Hefti and coworkers (1980) can be viewed as a model for changes that take place during Parkinson's disease (Hornykiewicz, 1979) and normal aging (Hornykiewicz, 1983). In both instances, decreases in DA levels are associated with increases in DA metabolites, suggesting increased turnover of DA by MAO.

Chapter 2: Materials and Methods

2.1 Materials

Male Swiss-Webster mice (25-35 grams) were obtained from Ace Animals and from Charles River (Crl:CFW (SW) BR). Care was taken not to mix breeders within a set of experiments. In addition, each experiment contained animals from a single shipment (i.e., same date of birth). In some experiments male preweanling mice (14-16 days) (Charles River, Swiss-Webster, 8-12 grams) were used. Mice were housed with a lactating mother (10 pups per mother).

Drugs and chemicals were obtained from the following sources: 6-OHDA, Regis Chemical Company; D,L-6-OH-DOPA, diethylenetriaminepentaacetic acid (DTPA), 3,4-dihydroxybenzylamine (DHB) hydrobromide, diethylmaleate (maleic acid diethyl ester, DEM), glutathione reductase, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), beta-nicotinamide adenine dinucleotide phosphate reduced form (NADPH), Sigma Chemical Company; L-ascorbic acid, monochloroacetic acid, acetonitrile (HPLC grade), and disodium EDTA, Fisher Scientific Company; perchloric acid (70 % solution in water) and sodium octylsulfate, Kodak; carbidopa (MK-486) Merck, Sharpe, and Dohme; reserpine (serpasil), Ciba Pharmaceutical Co.; pargyline HCl, Saber Laboratories Inc.; NSD-1015 (3-hydroxybenzylhydrazine, 2HCl), Research Biochemicals Inc.; desipramine HCl, USV Laboratories; nomifensine maleate, Hoechst-Roussel

Pharmaceuticals Inc.; mazindol, Sandoz Research Institute; tetrabenazine (RO1-9569), Hoffman LaRoche Inc.; and lubinol, Purepac Pharmaceutical Company.

2.2 Preparation of drugs for in vivo experiments

Note: See experiments for exact dose if several doses are given. A single dose indicates that the drug was given at that dose throughout all experiments.

6-OH-DOPA (100 or 200 mg/kg) was dissolved in a few drops of 2.0 M HCl and then diluted into 0.05 M HCl, containing 1.0 mM ascorbate. When carbidopa was used, it was dissolved together with the 6-OH-DOPA in a ratio of 1 part carbidopa to 4 parts 6-OH-DOPA, by weight (25 or 50 mg/kg). Pargyline (100 mg/kg), clorgyline (2.5 mg/kg) and nomifensine (40 mg/kg) were administered i.p. in 0.9 % (w/v) saline. Tetrabenazine (25 mg/kg) and reserpine (5, 7.5, or 10 mg/kg) were dissolved in 20 uL of glacial acetic acid and diluted into aqueous D-glucose (55 mg/L). NSD-1015 (100 mg/kg) and desipramine HCL (30 mg/kg) were dissolved in water. Diethylmaleate (2 x 1 mL/kg, 30 minute interval) was administered in lubinol.

All drugs were injected in a volume of 0.01 mL per gram of body weight. All injections were intraperitoneal (i.p.) with the exception of injection of NSD-1015, which was subcutaneous (s.c.).

2.3 Measurements of Catecholamines, Metabolites, 6-OH-DOPA, and 6-OHDA

At the termination of the experiments, mice were rapidly decapitated and brains were dissected over ice. Striatum was dissected according to the method of Glowinski and Iversen (1966). When frontal cortex was dissected, a 3 mm thick slice of brain was removed by making 2 coronal cuts, one at the level of the optic chiasm, another at the base of the olfactory bulb. From this slice, cortical tissue above the corpus callosum was collected.

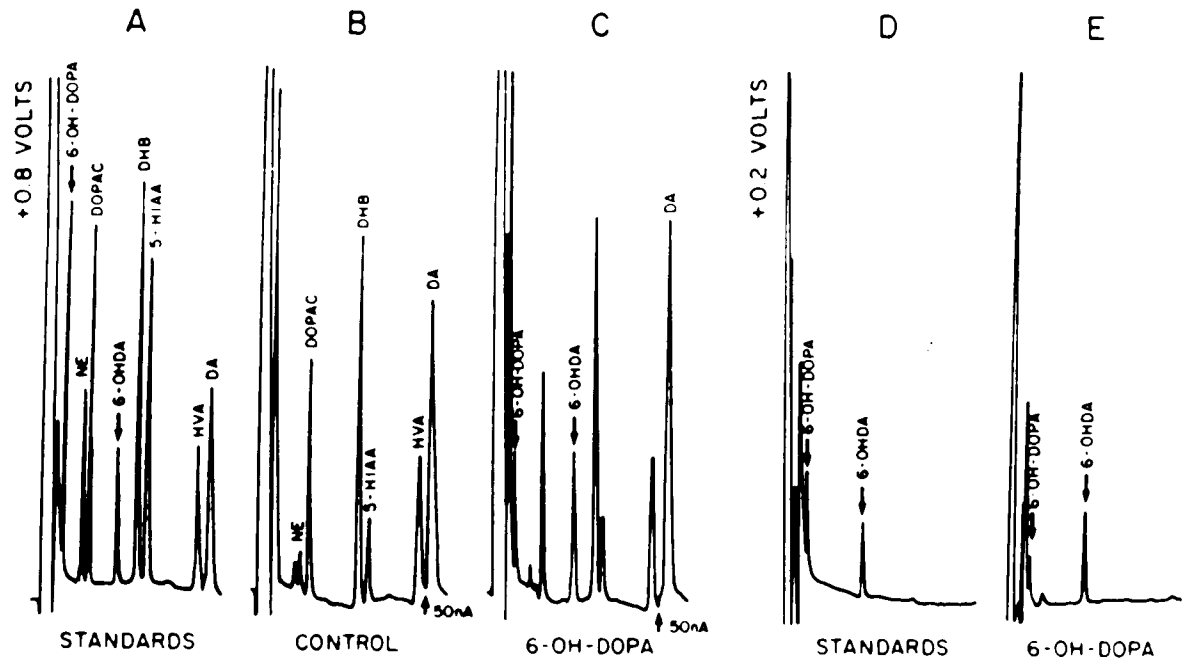
For routine catecholamine analysis, tissues were homogenized in 10 volumes of ice-cold 0.4 M perchloric acid (PCA), containing 0.1 mM ascorbic acid, 40 mg diethylenetriaminepenta-acetic acid (DTPA)/L, and 0.3 ug dihydroxybenzylamine (DHB)/mL (an internal standard). In experiments in which 6-OH-DOPA and 6-OHDA were measured, tissues were homogenized in 0.4 N PCA with 1.0 mM ascorbate, 40 mg/L DTPA, and 0.3 ug/mL DHB. When the accumulation of L-DOPA was measured, tissues were homogenized in 0.4 N PCA containing 40 mg/L DTPA and 0.3 ug/mL ascorbate. Analyses were routinely performed on the day that the tissues were collected (i.e., storage of samples was avoided). Homogenates were centrifuged (Hill scientific MV 15) for 10 min at 7,000 x g in order to precipitate proteins .

Supernatants were analyzed by high performance liquid chromatography with electrochemical detection (HPLC) with an

electrochemical detector equipped with a glassy-carbon electrode (Bioanalytical Systems, LC-4B amperometric detector, PM-30A dual piston pump). Because 6-OHDA and 6-OH-DOPA are easily oxidized, analyses were conducted at a low oxidizing potential of +0.2 V (versus a Ag/AgCl reference electrode), thereby allowing for selective visualization of these compounds without interference from other electrochemically-active tissue constituents, such as monoamines and metabolites. When tissue dopamine (DA) and norepinephrine (NE) were studied, a higher oxidizing potential of +0.8 V was used. However samples were also analyzed at +0.2 V to confirm the identity of the 6-OH-DOPA and 6-OHDA peaks. The sensitivity of the detector was set at either 5 or 10 nA/volt for full scale deflection of the chart recorder.

The mobile phase consisted of an aqueous solution of 0.15 M monochloroacetic acid, 0.7 mM EDTA, 2.0 mM sodium octylsulfate (as the paired ion), and 6.0 % acetonitrile; the pH was adjusted to 3.00 with NaOH pellets. The flow rate was 1.0 mL/min. Samples (50 uL) were injected over a C-18 reverse phase column (5 um beads, 25 cm length) (Biophase ODS). Sample peak heights and retention times were compared to those of known standards (5 ng).

Fig. 1 Representative chromatogram of standards and striata from control and 6-OH-DOPA-treated mice.



Chromatograms of standards and mouse striata. Panels A-C, detector set at +0.8 volts; panels D and E, detector set at +0.2 volts. In Panels B and C, the initial sensitivity of 10nA/Volt was decreased to 50 nA/Volt just prior to the emergence of the DA peak. Panel A: separation of standards (5 ng). Panel B: control animal. Panel C: at 30 minutes after the injection of 100 mg 6-OH-DOPA/kg. Panels D and E: same as panels A and C respectively except the detector was set at +0.2 volts.

2.4 Synaptosomal uptake of ^{3}H-DA and ^{3}H-NE

Synaptosomal uptake of ^{3}H-amines provides an index of cellular damage by measuring the functional integrity of the DA reuptake pump. This procedure is carried out according to the method of Berge et al. (1985) with slight modifications.

At the termination of the experiments, mice were rapidly decapitated and brains were dissected over ice. Striatum was dissected according to the method of Glowinski and Iversen (1966). When frontal cortex was dissected, a 3 mm thick slice of brain was removed by making 2 coronal cuts, one at the level of the optic chiasm, another at the base of the olfactory bulb. From this slice, cortical tissue above the corpus callosum was collected. Tissues were homogenized in 40 volumes (striatum) or 20 volumes (cortex) of ice cold 0.25M sucrose in a glass homogenizer with a teflon pestle. The homogenate was centrifuged (Hill Scientific MV-15) at 1000 x g for 10 minutes.

Aliquots of 75 uL of the supernatants were added to 600 uL of Krebs-phosphate buffer (pH 7.4) containing 11.8 mM NaCl, 5.6 mM D-glucose, 1.3 mM Na_2EDTA, 4.7 mM KCl, 1.8 mM $CaCl_2$-$2H_2O$, 1.2 mM $MgSO_4$, 16.2 mM Na_2HPO_4, and 15.9 mM $NaH_2PO_4 \cdot H_2O$, 1 mM ascorbate, and 1 uM pargyline HCl. In, addition some samples were incubated in the presence of 10 uM mazindol (striatum) or 10 uM desipramine (cortex) as a blank.

Samples were preincubated for 5 minutes at 37°C. Uptake was initiated by the addition of 25 uL of ^{3}H-DA or ^{3}H-NE at a final concentration of 5 or 20nM respectively. Incubations were terminated after one minute (uptake) or 10 minutes (storage) by the addition of 4 mL of ice-cold (0.9% w/v) saline to each tube. Samples were suction filtered and then washed with two successive 4 mL aliquots of saline, using a Brandel cell harvester (M-24R). Membranes were trapped on glass fiber filters (Schleicher and Schuell #32 glass 2X12 inch). Filters were placed in scintillation vials along with 10 mL of scintillation fluid (Liquiscint, National Diagnostics) and radioactivity was measured using liquid scintillation spectrometry (Beckman LS 3801). Specific uptake was measured as the total uptake less uptake in the presence of 10 uM mazindol (striatum) or 10 uM desipramine (cortex).

2.5 ^{3}H-Mazindol binding

^{3}H-Mazindol binding serves as an index of cellular damage by measuring changes in the number of DA reuptake sites. This procedure was carried out according to a modified version of the method of Javitch et al. (1984).

Animals were decapitated and striata were dissected over ice according to the method of Glowinski and Iversen (1966). Tissues were weighed and added to 80 volumes of buffer containing 120 mM NaCl, 5 mM KCl, 50 mM Tris HCl (pH 7.9 at 4°C). Striata were homogenized using a polytron

(Brinkmann Instruments Inc. PT 10/35) for a 10 second pulse. Homogenates were centrifuged at 25,000 x g for 20 minutes (Sorvall Superspeed RC2-B). Supernatants were discarded and the pellets were resuspended in the original volume of buffer using a polytron for a 5 second pulse. Centrifugation of and resuspension of tissue pellet were then repeated twice. Aliquots (200 uL) of this suspension were added to 25 uL of the same buffer or buffer containing unlabelled mazindol (a catecholamine uptake blocker) as a blank (final concentration 10 uM). Binding was initiated by the addition of 25 uL of ^{3}H-mazindol (4 nM final concentration). Binding was carried out at 4^{o}C for 1 hour.

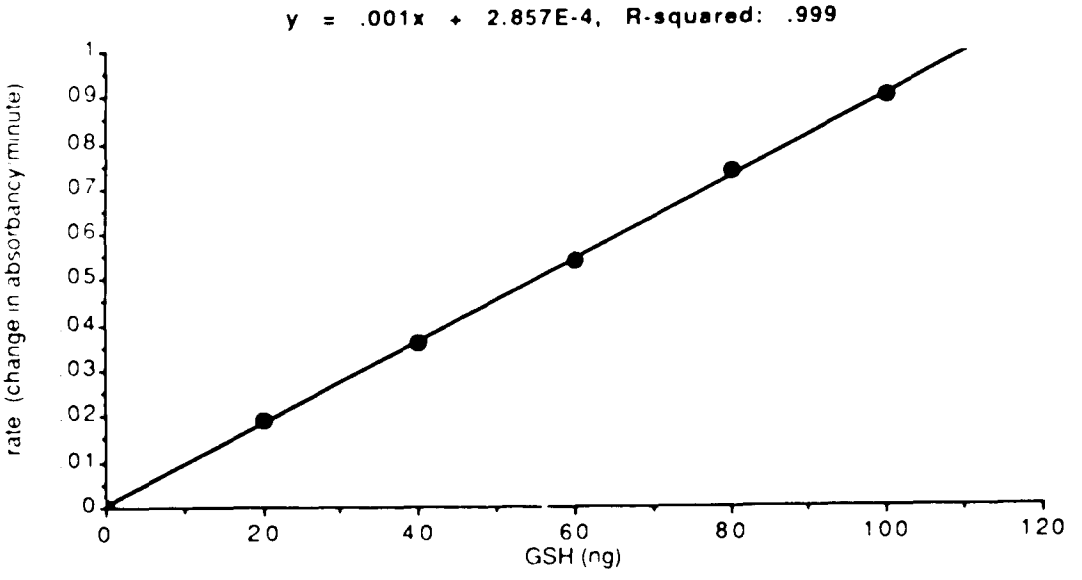
Binding was terminated by the addition of 4 mL of ice-cold buffer to each tube. Membranes were trapped by suction filtration (Brandel cell harvester, M24-R) on glass fiber filters (Schleicher and Schuell, #32 glass, 2 x 12 inch). Samples were subsequently washed with two successive 4 mL aliquots of buffer. Filters were placed in scintillation vials along with 10 mL of scintillation fluid (Liquiscint, National Diagnostics) and radioactivity was measured using liquid scintillation spectrometry (Beckman LS 3801). Specific binding was measured as the total binding less binding in the presence of 10 uM mazindol.

2.6 Measurement of tissue glutathione (GSH) levels

GSH was assayed by the enzymatic recycling procedure of Tietze (1969), as modified by Cooper et al. (1980). Mice were rapidly decapitated and striata were removed according to the method Glowinski and Iversen (1966). Tissues were weighed and homogenized in 10 volumes of 0.4 M perchloric acid with 40 mg/L of DTPA. Homogenates were centrifuged at 1,000 x g for 10 minutes. Immediately before assay, 50 uL of supernatant was added to 450 uL of buffer containing 100 mM KH_2PO_4 and 5 mM Na_2EDTA at pH 7.5. An aliquot (15 uL) of this diluted supernatant was further diluted with buffer to a final volume of 1.5 mL.

Spectrophotometric assays were performed according to the method of Cooper et al. (1980). Just prior to assay DTNB (200 uL) and NADPH (200 uL) were added to 1.5 mL of diluted sample. Assays were initiated by the addition of glutathione reductase (100 uL). Final concentrations for DTNB, NADPH, and glutathione reductase were 0.4mM DTNB, 0.17 mM NADPH, and 16 ug/mL glutathione reductase. The final assay volume was 2.0 mL. The rate of color formation (the rate of formation of 5-thio-2-nitrobenzoate) was monitored at 412 nM at room temperature for 5 minutes with a Stasar III flow-through spectrophotometer (Gilford Instruments). Rates of color formation were corrected for the blank rate (in the absence of GSH standard or sample), and compared to a standard curve of known amounts of GSH (Fig. 2).

Fig. 2 Standard curve for GSH



Each concentration was analyzed in duplicate. The standard error for each concentration was less than 5% of the mean.

2.7 Measurement of tyrosine hydroxylase activity

Tyrosine hydroxylase activity in striatum was measured in vivo according to the method of Nissbrandt and Carlsson (1987) and Nissbrandt et al. (1989). Tyrosine hydroxylase activity was assessed by the accumulation of L-DOPA at 30 minutes after administration of the centrally-acting DOPA-decarboxylase inhibitor NSD-1015 (3-hydroxybenzylhydrazine dihydrochloride, 100 mg/kg s.c.). Striata analyzed for levels of L-DOPA, DA, and HVA by chromatographic analysis as described in Section 2.3.

2.8 Statistical analyses

Statistical analyses were conducted by 2-tailed unpaired Student t-test. For multiple comparisons, data was analyzed by single factor analysis of variance (ANOVA), followed by either the Tukey test or Fisher's protected least significant difference (PLSD).

Chapter 3: Studies on the formation of 6-OHDA in mouse brain after systemic administration of 6-OH-DOPA

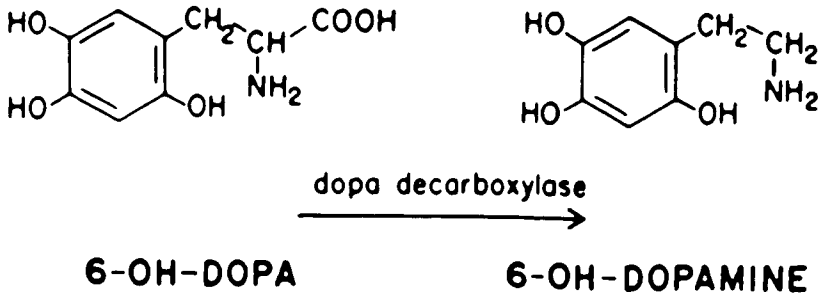
3.1 Introduction

Current evidence indicates that the neurotoxic action of 6-OH-DOPA is mediated by the formation of 6-OHDA (Ong et al., 1969; Corrodi et al., 1971; Sachs and Jonsson 1972a,b; Kostrzewa and Jacobowitz 1972, 1973; Figure 3). However, 6-OH-DOPA induced toxicity differs from that of 6-OHDA. Stereotaxic injection (i.c.v.) of 6-OHDA (100-500 ug) is frequently used to destroy both dopaminergic and noradrenergic neurons (Breese and Traylor, 1971; Iversen and Uretsky 1971; Kostrzewa and Jacobowitz, 1974). In contrast, 6-OH-DOPA, given either systemically or centrally (i.c.v) is selectively toxic to central noradrenergic nerve terminals, whereas central dopaminergic terminals, such as those of the striatum, nucleus accumbens, telencephalon, olfactory cortex, and hypothalamus, are spared (Corrodi et al., 1971; Jacobowitz and Kostrzewa 1971; Clarke et al., 1972; Sachs and Jonsson 1972a,b; Kostrzewa and Jacobowitz, 1973; Richardson and Jacobowitz, 1973; McClean et al. 1980, Kantak et al., 1981; and Cornwell-Jones and Bollers, 1983).

One obvious question is whether the resistance of dopaminergic neurons to 6-OH-DOPA can be explained by a lack of formation of 6-OHDA within dopaminergic terminals. This possibility was excluded by the observation that 6-OHDA was

present in high concentration in the striatum of mice after 6-OH-DOPA administration. The localization of 6-OHDA to storage vesicles was confirmed by comparing striatal levels of 6-OHDA in reserpine-pretreated mice to their respective controls. Additional experiments explore the interactions of 6-OH-DOPA with other drugs. These studies describe three independent ways by which brain levels of 6-OHDA can be elevated: inhibition of central 6-OHDA by MAO, inhibition of peripheral decarboxylase, and pretreatment with catecholamine uptake blockers.

Fig. 3 Conversion of 6-OH-DOPA to 6-OHDA

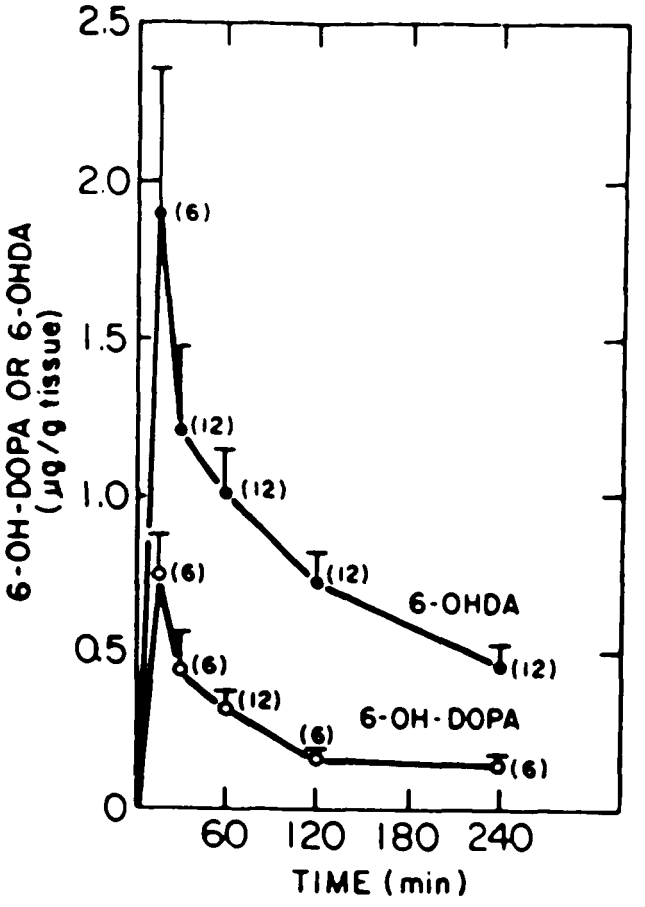


3.2 The presence of 6-OHDA in brain after systemic administration of 6-OH-DOPA

3.2.1 Time course experiments

Figure 4 shows the levels of 6-OH-DOPA and 6-OHDA found in the striatum over a 4 hour period following injection of 100 mg 6-OH-DOPA/kg. The 6-OHDA level was highest at 15 minutes, which was the earliest time point studied. At all time points, levels of 6-OHDA were higher than those of 6-OH-DOPA. Despite a known rapid rate of autoxidation of 6-OHDA in vitro, the neurotoxin was reasonably stable in the striatum over 4 hours. The time course indicated a more rapid loss between 15 and 30 minutes, followed by a slower decline thereafter. The slower decline showed a half-life of 2.4 hours. The time course of 6-OH-DOPA showed a similar pattern, maintaining concentrations 23-39% that of 6-OHDA throughout the 4 hour period.

Fig. 4 Time course of the appearance of 6-OH-DOPA and 6-OHDA in mouse striatum.

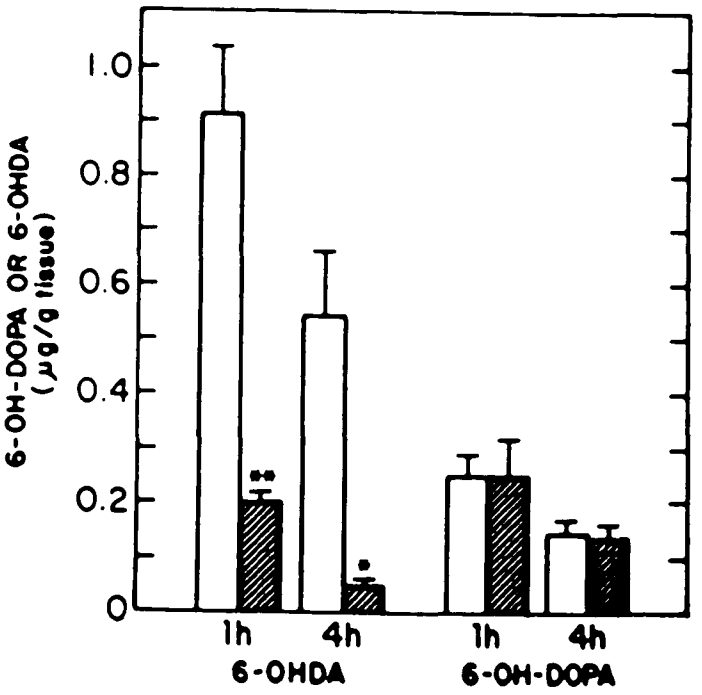


Levels of 6-OH-DOPA (open circles) and 6-OHDA (closed circles) in mouse striatum after systemic administration of 6-OH-DOPA (100 mg/kg i.p.) without pargyline or carbidopa.

3.2.2 Vesicular storage of 6-OHDA

Some animals were pretreated with reserpine (10 mg/kg, 1.5 hours) in order to inhibit the amine transport and storage mechanisms of synaptic vesicles. Fig. 5 shows that reserpine pretreatment significantly reduced the levels of 6-OHDA to 21.6% of control at 1 hour and 8.9% of control at 4 hours after the injection of 100 mg 6-OH-DOPA/kg. These results suggest that 6-OHDA is stored in synaptic vesicles. On the other hand, reserpine pretreatment did not significantly affect the levels of 6-OH-DOPA. This is as expected because reserpine blocks the storage of monoamines, but not their amino acid precursors, in synaptic vesicles. In the reserpine-pretreated group, levels of 6-OH-DOPA were equal to (1 hour) or greater than (4 hours) the levels of 6-OHDA, while in the absence of reserpine, 6-OH-DOPA was present at lower concentrations than 6-OHDA.

Fig. 5 6-OH-DOPA and 6-OHDA levels in striatum with and without pretreatment with reserpine



6-OHDA and 6-OH-DOPA in mouse striatum at 1 and 4 hours after injection of 6-OH-DOPA (100 mg/kg) without pargyline and carbidopa. Open bars = no reserpine (6-OH-DOPA alone); hatched bars = reserpine pretreated (10 mg/kg, 1.5 hours prior to 6-OH-DOPA). Results are pooled from two separate experiments and are expressed as mean $\pm$ SEM (n=6).
* $p < 0.005$ compared to 6-OH-DOPA alone
**$p < 0.001$ compared to 6-OH-DOPA alone
(Student t-test)

3.3 Interactions of 6-OH-DOPA with enzyme inhibitors and uptake blockers.

3.3.1 Inhibition of the peripheral decarboxylation of 6-OH-DOPA and inhibition of the metabolism of 6-OHDA by monoamine oxidase

Carbidopa, a peripheral DOPA-decarboxylase inhibitor, has been used by other investigators (e.g., Kostrzewa and Jacobowitz, 1973) to enhance the toxicity of 6-OH-DOPA. Carbidopa is typically used at a ratio of 1:4 compared to administered L-DOPA, to promote increased formation of DA from L-DOPA in brain (Physicians' Desk Reference, 1986). Pargyline at a dose of 100 mg/kg inhibits both MAO A and MAO B (Buu and Angers, 1987). Pargyline at this dose has been used in conjunction with 6-OH-DOPA in previous studies (VonVoigtlander and Losey, 1978). As shown in Table 1, pargyline and carbidopa were each effective in elevating levels of 6-OHDA in the striatum. When both were administered together, the effects were additive. This is expected since pargyline and carbidopa elevate amine levels via independent mechanisms.

Table 1. Effects of pargyline and carbidopa on levels of 6-OHDA in mouse striatum

Drugs	6-OHDA (ug/g ± SEM)		% Increase
6-OH-DOPA	0.486 ± 0.064	(n=18)	
6-OH-DOPA + pargyline	1.033 ± 0.130[a]	(n=13)	113 %
6-OH-DOPA + carbidopa	1.189 ± 0.130[b]	(n=14)	145 %
6-OH-DOPA + pargyline + carbidopa	1.752 ± 0.164[b,c]	(n=15)	261 %

6-OH-DOPA (100 mg/kg, i.p.) was injected alone or with carbidopa (25 mg/kg). Where indicated animals were pretreated with pargyline (100 mg/kg i.p.) 18 hours prior to 6-OH-DOPA. Striata were analyzed at 1 hour after 6-OH-DOPA.
Statistical analysis were performed with single factor ANOVA, followed by the Tukey test ($p < 0.0005$ across groups).
[a] $p < 0.025$ compared to 6-OH-DOPA alone
[b] $p < 0.005$ compared to 6-OH-DOPA alone
[c] $p < 0.025$ compared to 6-OH-DOPA with pargyline or carbidopa

3.3.2 Effect of uptake blockers on levels of 6-OHDA

Other workers have suggested that 6-OH-DOPA is decarboxylated extraneuronally to 6-OHDA which is in turn taken up into catecholamine terminals via the axonal membrane pump (Kostrzewa and Jacobowitz, 1973). This idea was based on the observation that pretreatment with agents known to block the uptake of NE into noradrenergic terminals, partially prevented the decrease in NE levels seen after 6-OH-DOPA administration (Kostrzewa and Jacobowitz, 1973). Similarly, VonVoigtlander and Losey (1978) were able to protect against decreases in brainstem NE and epinephrine by pretreating mice with selectively acting tricyclic antidepressants (agents known to act via the blockade of axonal uptake). However, methods to directly measure 6-OHDA were not available at that time.

Experiments were conducted to test whether pretreatment with uptake blockers would lower tissue levels of 6-OHDA by preventing the transport of 6-OHDA into noradrenergic nerve terminals. Table 2 shows experiments in which animals were pretreated with desipramine HCl (30 mg/kg, i.p.) 1 hour prior to injection of a neurotoxic dose of 6-OH-DOPA (see Chapter 4 for discussion of neurotoxic effects). A striking aspect of Table 2 is that desipramine markedly increased the levels of 6-OHDA in frontal cortex at both 1 hour (+125%) and 4 hours (+63%). These results seemed surprising and, therefore, a second "uptake blocker", nomifensine (40 mg/kg,

Table 2. Effect of desipramine on levels of 6-OHDA and NE in frontal cortex at 1 and 4 hours after a neurotoxic dose of 6-OH-DOPA.

	6-OHDA (ug/g ± SEM)	% Increase in 6-OHDA	NE (ug/g ± SEM)
1 hour			
6-OH-DOPA (n=8)	0.59 ± 0.15		0.31 ± 0.03
desipramine + 6-OH-DOPA (n=8)	1.33 ± 0.14[b]	125 %	0.28 ± 0.02
4 hours			
6-OH-DOPA (n=8)	0.52 ± 0.07		0.05 ± 0.01
desipramine + 6-OH-DOPA (n=8)	0.85 ± 0.07[b]	63 %	0.12 ± 0.03[a]

Animals were pretreated with desipramine HCl (30 mg/kg, i.p.) or vehicle 1 hour prior to co-injection of 6-OH-DOPA (200 mg/kg i.p., 2.5 hours) and carbidopa (50 mg/kg i.p.) or vehicle. All animals were pretreated with pargyline (100 mg/kg i.p., 18 hours). The results are from 2 independent experiments. For control data on the effects of desipramine by itself, see Table 4.

[a] $p < 0.02$ as compared to 6-OH-DOPA alone

[b] $p < 0.005$ as compared to 6-OH-DOPA alone

(Student t-test)

i.p., 40 minutes), was tested (Table 3). Nomifensine also elevated the levels of 6-OHDA in frontal cortex, and to a greater extent (+268% at 1 hour and +177% at 4 hours). These results show that, in contrast to expectations, uptake blockers actually elevate the levels of 6-OHDA found in the cortex after administration of 6-OH-DOPA.

Tables 2 and 3 also show levels of NE in frontal cortex. Kostrzewa and Jacobowitz (1973) had reported partial protection of NE levels, viewed at 24 hours after 6-OH-DOPA (100 mg/kg, i.v., without carbidopa or pargyline), when animals were pretreated with the same dose of desipramine HCl (30 mg/kg, i.p., 1 hour). Table 2 shows that a partial protection of NE levels was seen at 4 hours after 6-OH-DOPA (200 mg/kg, i.p., with carbidopa and pargyline), in agreement with Kostrzewa and Jacobowitz (1973). However, at 1 hour, the levels of NE were similar in animals receiving 6-OH-DOPA alone or desipramine plus 6-OH-DOPA. In control experiments, desipramine alone did not elevate levels of NE; indeed a significant decrease in NE was seen at 4 hours (Table 4). Therefore, desipramine does not protect NE stores by a direct action on NE levels. The latter results agree with Kostrzewa and Jacobowitz (1973) who also observed that desipramine by itself did not raise the levels of NE.

In the experiments with nomifensine (Table 3), no protection of NE levels was seen. Indeed nomifensine appeared to potentiate the loss of NE at 1 hour. In control

Table 3. Effect of nomifensine on the the levels of 6-OHDA and NE in frontal cortex at 1 and 4 hours after a neurotoxic dose of 6-OH-DOPA.

	6-OHDA (ug/g ± SEM)	% Increase in 6-OHDA	NE (ug/g ± SEM)
1 hour			
6-OH-DOPA (n=3)	0.58 ± 0.18		0.26 ± 0.02
nomifensine + 6-OH-DOPA (n=3)	2.02 ± 0.22[b]	248 %	0.14 ± 0.01[a]
4 hours			
6-OH-DOPA (n=4)	0.52 ± 0.09		0.08 ± 0.01
nomifensine + 6-OH-DOPA (n=4)	1.44 ± 0.05[c]	177 %	0.08 ± 0.01

Nomifensine (40 mg/kg, i.p.) or vehicle was administered 40 minutes prior to co-injection of 6-OH-DOPA (200 mg/kg i.p., 2.5 hours) and carbidopa (50 mg/kg i.p., 2.5 hours). All animals were pretreated with pargyline (100 mg/kg i.p., 18 hours). For control data on the effects of nomifensine by itself, see Table 4.

[a] $p < 0.02$ as compared to 6-OH-DOPA alone
[b] $p < 0.01$ as compared to 6-OH-DOPA alone
[c] $p < 0.001$ as compared to 6-OH-DOPA alone
(Student t-test)

Table 4. Effect of uptake blockers on levels of NE in the frontal cortex of mice.

	NE (ug/g ± SEM)
1 hour	
Control (n=6)	0.30 ± 0.01
Desipramine (n=6)	0.26 ± 0.02
Control (n=4)	0.39 ± 0.02
Nomifensine (n=3)	0.05 ± 0.02[b]
4 hours	
Control (n=6)	0.29 ± 0.02
Desipramine (n=6)	0.22 ± 0.02[a]
Control (n=4)	0.38 ± 0.01
Nomifensine (n=4)	0.26 ± 0.01[b]

All animals were pretreated with pargyline (100 mg/kg i.p., 18 hours). Where indicated animals received desipramine HCl (30 mg/kg i.p., 1 hour) or nomifensine (40 mg/kg i.p., 40 minutes). Samples were analyzed for tissue levels of NE at 1 and 4 hours after the pretreatment period.

[a] $p < 0.05$ as compared to control

[b] $p < 0.001$ as compared to control

(Student t-test)

experiments (Table 4), nomifensine markedly decreased the level of NE at 1 hour (-87%, $p < 0.01$). The nomifensine-induced decrease in NE levels was short lived and had nearly recovered by 4 hours. This observation is in agreement with a known releasing action of nomifensine on NE in cortical noradrenergic terminals (Braestrup and Scheel-Kruger, 1976; Racagni et al., 1982a,b; Wood et al., 1986).

As noted previously, there was no apparent effect of desipramine on levels of NE at one hour after 6-OH-DOPA (Table 2). Comparison of the data in Table 2 with control data in Table 4 would make it appear that there had not been an effect of 6-OH-DOPA alone at 1 hour (0.30 ug NE/g for control mice, Table 4, vs. 0.31 ug/g for 6-OH-DOPA-treated mice, Table 2). However, such a comparison would not be fair because these were separate experiments with different shipments of mice. A separate experiment was run to assess the effect of 6-OH-DOPA at one hour. It was observed that levels of NE were somewhat decreased at 1 hour after 6-OH-DOPA (from 0.32 $\pm$ 0.01 (SEM) ug/g to 0.23 $\pm$ 0.01 ug/g (n=5), $p < 0.001$ Student t-test).

Since uptake blockers elevate the levels of 6-OHDA in the cortex (Tables 2 and 3), it was of interest to study the striatum also. For comparison with previous striatal data (Figs. 4 and 5), similar conditions were used (100 mg 6-OH-DOPA/kg, without carbidopa or pargyline). Levels of 6-OHDA in striatum at 4 hours in nomifensine pretreated mice were 85% greater ($p < 0.02$) than those of animals receiving 6-OH-

DOPA alone (Table 5). However, 6-OHDA levels were similar in both groups at 1 hour. Levels of 6-OH-DOPA were significantly lower in nomifensine-pretreated animals at 1 hour.

In the course of these experiments, it was noted that cortical 6-OHDA levels were essentially unchanged between 1 and 4 hours (Tables 2 and 3), in contrast to results in the striatum (Fig. 4), where a substantial decline was noted between 1 and 4 hours. However, other experimental factors such as dose of 6-OH-DOPA (100 mg/kg in Fig.4, 200 mg/kg in Tables 2 and 3) or the presence (Tables 2 and 3) or absence (Fig. 4) of pargyline or carbidopa, could have played a role. To obtain a better comparison with Tables 2 and 3 (cortex), an experiment was performed to analyze striatal levels of 6-OHDA at 1 and 4 hours under the same conditions as Tables 2 and 3 (200 mg 6-OH-DOPA/kg with pargyline and carbidopa). The results showed that under these conditions, 6-OHDA was not unstable; 6-OHDA levels were 2.93 $\pm$ 0.41 ug/g (SEM) at 1 hour and 3.89 $\pm$ 0.27 ug/g at 4 hours (n=6 per group, not significantly different, $p > 0.05$, Student t-test). Over the same time period, 6-OH-DOPA levels fell from 0.46 $\pm$ 0.09 ug/g at 1 hour to 0.20 $\pm$ 0.01 ug/g at 4 hours ($p < 0.02$).

Table 5. Effect of nomifensine-pretreatment on the levels of 6-OH-DOPA and 6-OHDA in striatum after administration of 6-OH-DOPA.

	6-OHDA (ug/g ± SEM)	% Increase 6-OHDA	6-OH-DOPA (ug/g ± SEM)
1 hour			
6-OH-DOPA (n=8)	1.06 ± 0.06		0.21 ± 0.02
nomifensine + 6-OH-DOPA (n=8)	1.08 ± 0.19	2 %	0.13 ± 0.02[a]
4 hours			
6-OH-DOPA (n=8)	0.62 ± 0.07		0.12 ± 0.06
nomifensine + 6-OH-DOPA (n=8)	1.15 ± 0.17[b]	85 %	0.06 ± 0.01

Levels of 6-OH-DOPA and 6-OHDA at 1 and 4 hours after 6-OH-DOPA (100 mg/kg i.p.). Where indicated animals received nomifensine (40 mg/kg, i.p.) 40 minutes prior to 6-OH-DOPA.
[a] $p < 0.05$ as compared to 6-OH-DOPA alone
[b] $p < 0.02$ as compared to 6-OH-DOPA alone
(Student t-test)

3.4 Discussion

This work represents the first description of the levels of 6-OH-DOPA and 6-OHDA in brain after systemic administration of 6-OH-DOPA. The presence of relatively high concentrations of 6-OHDA in the striatum, an area receiving a dense dopaminergic innervation, indicates that the resistance of dopaminergic terminals to 6-OH-DOPA cannot be attributed to a lack of formation of 6-OHDA in the striatum. In addition, the experiments with reserpine show that the 6-OHDA is stored within DA terminals. The possibility remains that dopaminergic neurons differ from noradrenergic neurons in their sensitivity to 6-OH-DOPA by virtue of some unique protective mechanism. This idea will be explored further in Chapter 5.

Despite a known rapid autoxidation of 6-OHDA at neutral pH in vitro (Heikkila and Cohen, 1972; Graham et al., 1978), the 6-OHDA generated in brain appeared fairly stable in the presence of uninhibited MAO. A number of factors can contribute to the observed stability of 6-OHDA: storage in synaptic vesicles, continued synthesis of 6-OHDA from precursor amino acid, and redox cycling of the o- and p-quinones of 6-OHDA. A direct test for an interaction with amine storage vesicles was performed with reserpine, which blocks the uptake of amines into synaptic vesicles. Reserpine (10 mg/kg) induced a marked lowering of 6-OHDA in the striatum (Fig. 5). Previously, Jonsson and Sachs (1971)

reported that reserpine (10 mg/kg, i.p.) interfered with the uptake in vitro of tritium-labeled 6-OHDA by sympathetic nerves in mouse atria. Therefore, storage in synaptic vesicles is indicated. Binding in vesicles may make the 6-OHDA less subject to autoxidation.

It should be noted that the levels of 6-OH-DOPA were much lower than 6-OHDA (Fig. 4). Therefore, the contribution from continued decarboxylation of 6-OH-DOPA would be relatively small. However, in the reserpinized state, tissue levels of 6-OH-DOPA were equal to or greater than 6-OHDA (Fig. 5) and, therefore, the persistence of low levels of 6-OHDA may reflect, in part, continued decarboxylation. The presence of detectable levels of 6-OH-DOPA for up to 4 hours may reflect its existence in regions that do not contain the L-aromatic amino acid decarboxylase.

Autoxidation of 6-OHDA leads to the formation of o- and p-quinones that undergo addition reactions with glutathione; a glutathione adduct has been reported in brain after intracerebral injection of 6-OHDA (Liang et al., 1977). Another likely possibility is that the o- and p-quinones are recycled to 6-OHDA by reaction with tissue ascorbate (Heikkila & Cohen, 1972). Injection (i.p.) of the o- and p-quinones of 6-OHDA into mice leads to destruction of peripheral sympathetic nerve terminals (Heikkila et al., 1973), which implies reduction to 6-OHDA in order to permit transport into catecholamine neurons via the axonal membrane pump. Nonetheless, the strong effect of reserpine (Fig.5)

indicates that vesicular storage plays the major role in stabilizing 6-OHDA in the CNS.

Pretreatment with inhibitors of MAO, such as pargyline or nialamide, potentiates the neurodegenerative effects of 6-OH-DOPA (Kostrzewa and Jacobowitz, 1972 and 1973; Sachs and Jonsson, 1972a and 1972b; VonVoigtlander and Losey, 1978) and 6-OHDA (Breese and Traylor, 1971; Kostrzewa and Jacobowitz, 1974). An implication is that 6-OHDA is metabolized, in part, by MAO and, therefore, the inhibitors enhance the levels of 6-OHDA in tissues. This idea is supported by a study by Jonsson and Sachs (1971) which provides evidence for the metabolism of 6-OHDA by MAO. In the current study, pargyline pretreatment enhanced 6-OHDA levels in the striatum by 113% at 1 hour after 100 mg 6-OH-DOPA/kg (Table 1). Similarly, carbidopa, a peripherally-acting inhibitor of DOPA-decarboxylase potentiates the toxic effects of 6-OH-DOPA on central noradrenergic neurons (Kostrzewa and Jacobowitz, 1973). In the current study, carbidopa increased the levels of 6-OHDA by 145% in the striatum (Table 1). These results suggest that the increased neurotoxicity seen after treatment with pargyline or carbidopa is mediated by an increase in neuronal levels of 6-OHDA.

Since 6-OHDA is formed by decarboxylation and is subsequently stored in reserpine-sensitive vesicles, it is of interest to note the localization of the L-aromatic amino

acid decarboxylase in the striatum. Studies by Melamed et al. (1981) and Hefti et al. (1981) in rats lesioned with 6-OHDA have indicated that 80-85% of the decarboxylase in the striatum is present in nigrostriatal nerve terminals. A small, but significant portion (10-15%) is also present in non-aminergic neurons that can be lesioned with kainic acid, while serotonin terminals do not make a detectable contribution to the total level of decarboxylase. Following the lesioning of nigrostriatal neurons to reduce striatal DA and tyrosine hydroxylase by 95%, functional DA is still produced from exogenously administered L-DOPA (Melamed et al., 1981; Hefti et al., 1981).

The localization of the decarboxylase to nigrostriatal terminals would seem to indicate that this is the major site of synthesis of 6-OHDA. However, this interpretation is in apparent conflict with the suggestion by Kostrzewa and Jacobowitz (1973) that partial protection of NE levels, observed when animals are pretreated with uptake blockers prior to 6-OH-DOPA administration, is due to inhibition of 6-OHDA transport across the axonal membrane.

6-OHDA is transported into catecholamine neurons via the energy dependent axonal membrane pump (Jonsson and Sachs, 1970, 1971). Pretreatment with uptake blockers prevents a portion of the decrease in whole brain NE after intracisternal administration of 6-OHDA (Breese and Traylor, 1971). In contrast, transport of amino acids such as L-DOPA and 6-OH-DOPA, into catecholamine terminals is independent

of the membrane pump, and would not be expected to be affected by pretreatment with uptake blockers. Therefore, pretreatment with uptake blockers would be expected to prevent 6-OH-DOPA induced toxicity only if 6-OH-DOPA is largely decarboxylated extraneuronally. Similarly, if 6-OH-DOPA is decarboxylated extraneuronally, then levels of 6-OHDA in catecholamine terminals in animals pretreated with uptake blockers would be expected to be decreased.

Levels of 6-OHDA in animals pretreated with uptake blockers prior to administration of 6-OH-DOPA were remarkably greater than those of their respective controls in both the cortex (Tables 2 and 3) and the striatum (Table 5). These data strongly suggest that the decarboxylation of 6-OH-DOPA takes place mainly within dopaminergic nerve terminals. This is in agreement with the strong localization of DOPA-decarboxylase to the terminals of nigrostriatal neurons. Moreover, experiments with reserpine localize 6-OHDA to synaptic vesicles within catecholamine terminals, and argue against the possibility of storage of 6-OHDA outside of catecholamine terminals.

One possible explanation for the increased levels of 6-OHDA in animals pretreated with uptake blockers may involve the known inhibitory effects of these drugs on the firing rates of central catecholamine neurons. Single unit recordings from noradrenergic neurons in the locus ceruleus demonstrated decreases in the firing rates of these cells

almost immediately after intravenous administration of desipramine and other tricyclic antidepressants (Nyback et al., 1975; Scuvee-Moreau and Dresse, 1979). In experiments with rats, desipramine (30 mg/kg i.p.) caused a complete cessation of firing of all locus ceruleus neurons tested (Olpe et al., 1983). Administration of the alpha-adrenergic antagonist, piperoxane, tended to reverse the decrease in firing rate observed after desipramine (Haskins et al., 1985).

Antidepressants, are also known to decrease the turnover of NE and serotonin in brain (Corrodi and Fuxe, 1969; Schubert et al., 1970). Decreases in turnover are most likely due to an increased transmitter-receptor interaction and consequent receptor-mediated feedback inhibition.

Scuvee-Moreau and Dresse (1979) outline 3 possible mechanisms for regulation of noradrenergic neurotransmission: These include (1) an increased stimulation of post-synaptic receptors which would decrease the activity of locus ceruleus neurons indirectly via a multineuronal feedback loop; (2) activation of $alpha_2$-autoreceptors on locus ceruleus neurons, which may involve inhibitory noradrenergic collaterals (Aghajanian and Cedarbaum, 1977; Cedarbaum and Aghajanian, 1977); or (3) increased activation of presynaptic $alpha_2$-adrenergic autoreceptors which would tend to decrease neurotransmitter release.

Similarly, single unit recordings from neurons in the substantia nigra of mice revealed a decrease in the firing rate of nigral neurons in animals pretreated with the antidepressant drug, nomifensine (20-36 mg/kg s.c.) (Studer and Schultz, 1987). The dopamine receptor blocker, haloperidol (0.4-0.7 mg/kg), reversed the nomifensine-induced decreases in firing rates in all neurons. The exact mechanisms by which firing rates of nigral neurons are modulated are not clear. In addition to the terminals in the striatum, evidence also exists for uptake and release at the somatodendritic level in the nigra (Bjorklund and Lindvall, 1975; Nieoullon, et al., 1977). Moreover, a known direct DA-releasing action of nomifensine must also be considered (Braestrup and Scheel-Kruger, 1976; Wood et al., 1986).

Experiments with reserpine (Fig. 5) indicate that 6-OHDA is stored in vesicles. Decreases in firing rates associated with pretreatment with uptake blockers may increase this pool of 6-OHDA by decreasing exocytotic release of 6-OHDA stored in vesicles. Therefore, higher levels of 6-OHDA in desipramine-treated (Table 2) and nomifensine-treated (Table 3) mice as compared to mice receiving 6-OH-DOPA alone, may be due to suppression of the firing rate of locus ceruleus neurons by desipramine or nomifensine. The more pronounced effect of nomifensine, as compared to desipramine, may reflect its increased potency

in inhibiting the firing rates of locus ceruleus neurons: The mean total doses required to produce a 50% inhibition of firing rate of locus ceruleus neurons was approximately 4-fold greater for desipramine (0.29 mg/kg) as compared to nomifensine (0.07 mg/kg) (Scuvee-Moreau and Dresse, 1982). These data suggest that the greater effect of nomifensine on cortical 6-OHDA levels may be related to its greater efficacy in inhibiting the firing rate of locus ceruleus neurons.

Unlike desipramine, nomifensine did not protect against 6-OH-DOPA-induced decreases in NE levels at 4 hours. The acute decrease in NE levels seen at 1 hour after nomifensine (Table 4) is consistent with its known releasing action at cortical noradrenergic terminals (Braestrup and Scheel-Kruger, 1976; Racagni et al., 1982a,b; Wood et al., 1986,). The lack of protection of NE levels at 4 hours by nomifensine may also be related to amount of 6-OHDA formed. The amount of 6-OHDA in nomifensine-pretreated mice was 1.5 times (1 hour) and 1.7 times (4 hours) greater than that seen in desipramine-pretreated animals. These data suggest that protection may not be possible in the presence of higher concentrations of 6-OHDA.

The mechanism of protection of 6-OH-DOPA-induced depletions in NE levels by uptake blockers is not clear. Results from control experiments (Table 4) indicate that desipramine does not directly elevate levels of NE. However, the presence of sizable quantities of 6-OHDA in

both the frontal cortex and striatum of animals pretreated with uptake blockers indicates that the mechanism of protection is certainly not the interruption of transport of 6-OHDA into catecholamine nerve terminals, as suggested by Kostrzewa and Jacobowitz (1973). The mechanism of protection may instead involve the marked decreases in firing rate invoked by these drugs. Decreases in firing rates in conjunction with an ongoing neurodegenerative event may lead to increased levels of catecholamine in some but not all cases.

These data provide direct evidence that the original interpretation, by Kostrzewa and Jacobowitz (1973), that uptake blockers protect against 6-OH-DOPA-induced decreases in NE levels by preventing the transport of extraneuronally-derived 6-OHDA across the axonal membrane, is incorrect. Experiments here, based on a direct assay of tissue levels of 6-OHDA, show that uptake blockers actually increase levels of 6-OHDA.

Jonsson and Sachs (1971) reported that 6-OHDA is a substrate for MAO. This is confirmed in the current study by the observation (Table 1) that inhibition of MAO increases the levels of 6-OHDA. While inhibition of MAO potentiates the toxic effects of 6-OH-DOPA (Sachs and Jonsson, 1972a), inhibition of COMT does not (Kostrzewa and Jacobowitz, 1972, 1973), indicating that 6-OHDA is probably not a substrate for the latter enzyme. The latter

observation appears to be confirmed in the current study by the observation that 6-OHDA levels in both cortex and striatum were stable between 1 and 4 hours after 6-OH-DOPA in pargyline-treated animals. Thus, this study shows that storage in vesicles and MAO activity are two critical factors affecting the stability of 6-OHDA in brain.

Chapter 4: Neurotoxic effects of 6-OHDA derived from 6-OH-DOPA

4.1 Introduction

The experiments in Chapter 3 describe some the of the properties of 6-OHDA derived metabolically from 6-OH-DOPA (i.e., stability, localization, decarboxylation, and interactions with pharmacological agents). Chapters 4 and 5 will focus on the neurotoxic effects of 6-OH-DOPA. Chapter 4 confirms the known selective toxicity and extends this observation with information on the levels of 6-OHDA in brain areas known to be either sensitive or resistant to 6-OH-DOPA induced toxicity. Chapter 5 explores several possible routes for potentiation of the neurodegenerative effects of 6-OH-DOPA.

4.2 Levels of 6-OHDA, NE, and DA in mouse brain after 6-OH-DOPA administration

Other investigators have shown that systemic administration of 6-OH-DOPA destroys noradrenergic terminals in the cortex, while dopaminergic terminals in the striatum are spared (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson, 1972a,b). Levels of 6-OHDA and catecholamines in the striatum and frontal cortex at 2.5 hours after the administration of 200 mg 6-OH-DOPA/kg (with pargyline and carbidopa) are shown in Table 6. High levels of 6-OHDA seen in the striatum correspond to its rich innervation by

dopaminergic terminals; the molar level of 6-OHDA was 56% that of endogenous DA in control animals. 6-OHDA in the striatum was 8.8-fold higher than in the frontal cortex. NE in the cortex was reduced by 77% ($p < 0.001$). In the striatum, on the other hand, DA levels were not affected by 6-OH-DOPA, while NE levels were decreased by only 28% (from 0.40 ± 0.05 (SEM); n=12 ug/g to 0.29 ± 0.03 ug/g, $0.1 > p > 0.05$).

Table 6. Levels of NE, DA, and 6-OHDA in frontal cortex and striatum after systemic administration of 6-OH-DOPA.

Brain Area	Treatment	Brain Amine Level (ug/g ± SEM)	
		NE	6-OHDA
Cortex	Control	0.53 ± 0.03 (n=29)	0.00 ± 0.00 (n=29)
	6-OH-DOPA	0.12 ± 0.03 (n=29)[a]	0.63 ± 0.06 (n=17)[a]
		DA	6-OHDA
Striatum	Control	9.00 ± 0.53 (n=12)	0.00 ± 0.00 (n=12)
	6-OH-DOPA	9.40 ± 0.46 (n=12)	5.54 ± 0.42 (n=12)[a]

All animals were pretreated with pargyline (100 mg/kg, 18 h). 6-OH-DOPA (200 mg/kg) was co-injected with carbidopa (50 mg/kg). Data are at 2.5 h after 6-OH-DOPA.
[a] $p < 0.001$ compared to control (Student t-test)

4.3 Effects of 6-OH-DOPA administration on synaptosomal uptake of ^{3}H-catecholamines

Synaptosomal uptake of ^{3}H-DA (striatum) and ^{3}H-NE (frontal cortex) was used as an additional index of toxicity in order to confirm the differential effect of 6-OH-DOPA on neurotransmitter levels. 6-OH-DOPA (100 mg/kg i.p. with pargyline and carbidopa) caused a 74.1% ($p < 0.001$) decrease in the uptake of ^{3}H-NE into cortical synaptosomes at 2.5 hours after 6-OH-DOPA (Table 7). A 2-fold increase in the dose of 6-OH-DOPA (200 mg/kg i.p. with pargyline and carbidopa) did not produce a further reduction in uptake (72.1% $p < 0.001$), suggesting that a maximal toxic effect is achieved by the lower dose. 6-OH-DOPA (200 mg/kg i.p. with pargyline and carbidopa) failed to alter the uptake of ^{3}H-DA into striatal synaptosomes at 2.5, 5, or 24 hours after 6-OH-DOPA (Table 8). A relatively small decrease in uptake of ^{3}H-DA at 2.5 hours did not achieve statistical significance and had recovered by 5 hours. These results are consistent with the well known resistance of dopaminergic nerve terminals to 6-OH-DOPA.

Table 7. Effect of pretreatment of mice with 6-OH-DOPA on the uptake of ^{3}H-NE into cortical synaptosomes.

	6-OH-DOPA (100 mg/kg) CARBIDOPA (25 mg/kg) (A)	6-OH-DOPA (200 mg/kg) CARBIDOPA (50 mg/kg) (B)
	(cpm x 10^2 ± SEM)*	
Control (n=6)	28.5 ± 4.4	16.5 ± 1.9
6-OH-DOPA (n=6)	7.4 ± 1.9[a] (25.9%)	4.6 ± 0.9[a] (27.9%)

Uptake and storage of ^{3}H-NE was carried out for a 10 minute period. Values in parentheses are percent control. 6-OH-DOPA (2.5 hours) and carbidopa were co-injected as indicated. All animals were pretreated with pargyline (100 mg/kg i.p.) 18 hours prior to 6-OH-DOPA or vehicle. The data are from 2 independent experiments at each dose.

*Results with 100 mg 6-OH-DOPA/kg (column A) were obtained with 3.57 mg of original tissue incubated with 20 nM ^{3}H-NE. Results with 200 mg 6-OH-DOPA/kg (column B) were obtained with 6.82 mg of original tissue incubated with 10 nM ^{3}H-NE. In (A), tissue from one animal was homogenized in 20 volumes of 0.25M sucrose; in (B), tissue from two animals was homogenized in 10 volumes of sucrose.

[a] $p < 0.001$ as compared to control (Student t-test)

Table 8. Effect of pretreatment of mice with 6-OH-DOPA on the uptake of ^{3}H-DA into striatal synaptosomes.

	cpm x 10^3 ± SEM
Control (n=13)	36.4 ± 2.5
6-OH-DOPA 2.5h (n=7)	30.0 ± 2.9 (82.4%)
6-OH-DOPA 5 h (n=3)	41.1 ± 3.5 (112.9%)
6-OH-DOPA 24 h (n=3)	39.4 ± 5.3 (108.2%)

Uptake and storage of ^{3}H-DA was carried out for a 10 minute period. Values in parentheses are percent control. Data are the observed radioactivity accumulated by 1.83 mg of original tissue. 6-OH-DOPA (200 mg/kg i.p.) was administered with carbidopa (50 mg/kg) to pargyline-pretreated (100 mg/kg i.p., 18 hours) mice. Control values are pooled over 4 independent experiments. 6-OH-DOPA 2.5 hour data are from 2 independent experiments.
Statistics were performed by single factor ANOVA ($p=0.1972$ across groups)

4.4 Discussion

The administration of a neurotoxic dose of 6-OH-DOPA markedly lowered levels of NE in the frontal cortex, while levels of DA in the striatum remained unchanged (Table 6). Yet levels of 6-OHDA were 8.8-fold higher in the striatum than in the frontal cortex (Table 6). It is not clear however, that dopaminergic terminals in the striatum are necessarily exposed to higher concentrations of 6-OHDA. The innervation of the striatum from the substantia nigra is more dense than the innervation of the frontal cortex from the locus ceruleus. Therefore, it is conceivable that the 6-OHDA in the striatum is dispersed over a much larger number of nerve terminals, yielding a similar concentration of 6-OHDA in both dopaminergic and noradrenergic terminals. However, studies directed at the effects of 6-OH-DOPA on dopaminergic neurons in different brain areas seem to indicate that the resistance of dopaminergic neurons to 6-OH-DOPA can be generalized to other DA systems (i.e., mesolimbic and olfactory).

When dopaminergic and noradrenergic nerve terminals are present to a similar extent within the same brain area, such as the olfactory cortex (Cornwell-Jones and Bollers, 1983) or the hypothalamus (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson, 1972a,b; Richardson and Kostrzewa, 1973), the resistance of dopaminergic terminals to 6-OH-DOPA persists. Cornwell-Jones and Bollers (1983) observed a 50% decrease

in levels of NE in the olfactory cortex of adult (postnatal day 81-93) male rats that had been injected with 6-OH-DOPA (60 ug/g i.p.) at birth, while DA levels in the same samples remained unchanged. Similarly, in the hypothalamus, the dense innervation of the median eminence by the dopaminergic neurons of the arcuate nucleus displayed unaltered histofluorescence; in contrast, histofluorescence of noradrenergic cell bodies and terminals of the hypothalamus (periventricular, paraventricular, and dorsomedial nuclei, as well as the internal layer of the median eminence) was noticeably reduced (Jacobowitz and Kostrzewa, 1971; Sachs and Jonsson, 1972a,b; Richardson and Jacobowitz, 1973).

These observations argue for the existence of intrinsic differences between dopaminergic and noradrenergic terminals. These might include: 1, differential binding of 6-OHDA in dopaminergic versus noradrenergic vesicles; 2, differences in levels of superoxide dismutase and cytosolic catecholamines, which inhibit the rate of production of hydrogen peroxide during the autoxidation of 6-OHDA (Sachs et al., 1975; Cohen & Heikkila, 1977); and 3, differences in cellular protective mechanisms against peroxide-mediated cell damage.

Whereas NE in the cortex was markedly diminished by 6-OH-DOPA (Table 6), NE in the striatum was only moderately decreased (text). The weaker effect on striatal NE is most likely due to a lack of noradrenergic terminals in the striatum: Fluorescence microscopy (Jacobowitz, 1973) and

autoradiographic studies with ^{3}H-DA uptake and selective monoamine uptake blockers in control and 6-OHDA-lesioned animals (Doucet et al., 1986), indicate that NE measured in the striatum is probably present largely in fibers originating in the locus ceruleus and other pontine nuclei, and coursing through the striatum to the frontal cortex. In that event, the levels of DOPA-decarboxylase may be low and the uptake mechanism (for preformed 6-OHDA) may not be as effective compared to nerve terminals in the cortex.

Changes in synaptosomal uptake after 6-OH-DOPA (Tables 7 and 8) correlated with the effects of 6-OH-DOPA on neurotransmitter levels (Table 6). The uptake of ^{3}H-NE into cortical synaptosomes was reduced by approximately 73% when 6-OH-DOPA was administered with either 100 mg/kg or 200 mg/kg (Table 7); however, the uptake of ^{3}H-DA into striatal synaptosomes was not significantly affected by the higher dose of 6-OH-DOPA (Table 8).

The similarity between changes in neurotransmitter levels and uptake of ^{3}H-amines has been previously described by Sachs and Jonsson (1972a,b). Uptake of ^{3}H-MA into cortical slices was reduced 65% at 16 hours after 6-OH-DOPA (3 x 100 mg/kg with nialamide pretreatment 100 mg/kg i.p., 2 hours) and had not recovered by 42 days. Decreases in whole brain NE paralleled the effects of on uptake. At the same time, there was no change in the uptake of ^{3}H-MA into striatal slices. Similarly, uptake of ^{3}H-DA at one week

after 6-OH-DOPA was also unchanged (Sachs and Jonsson, 1972a).

Seiden and Vosmer (1984) have suggested that 6-OHDA, formed in relatively small amounts after injection of methylamphetamine, might be responsible for the observed neurodegenerative effects of methylamphetamine on dopaminergic terminals in the striatum. The mean levels of 6-OHDA reported in the striatum of rats after injection of methylamphetamine (Seiden and Vosmer, 1984) were 0.20 ug/g at 30 minutes, 0.39 ug/g at 1 hour, and 0.24 ug/g at 2 hours. 6-OHDA was not detected beyond 2 hours in the latter study. The proposed mechanism is release of DA from storage sites, followed by non-enzymatic oxidation of DA in the synapse. The hydroxylation of DA in vitro by model H_2O_2 and hydroxyl radical generating systems has been reported (Senoh et. al., 1959; Slivka and Cohen, 1985). Products include 2-, 5-, and 6-OHDA in a ratio of 3:2:1, and smaller amounts of NE (Slivka and Cohen, 1985). However, Rollema et. al. (1986) failed to confirm 6-OHDA in the striatum after administration of methylamphetamine. Similarly, in this laboratory, 6-OHDA (as well as 2- and 5-OHDA) were not observed in the striatum of mice after administration of methylamphetamine (Slivka & Cohen, unpublished observation). Although it remains uncertain whether the autoxidation of DA in vivo can lead to formation of 6-OHDA, the likelihood that endogenously produced 6-OHDA induces the destruction of striatal DA terminals by methylamphetamine is questionable.

The amount of 6-OHDA reported in the striatum after methylamphetamine (Seiden and Vosmer, 1984) is less than 5% that observed after 200 mg 6-OH-DOPA/kg (Table 9).

Table 9. 6-Hydroxydopamine in brain after administration of 6-OH-DOPA or methylamphetamine.

Treatment	Brain Amine Level (ug/g ± SEM)		Source
	6-OHDA	Dopamine	
Control (n=12)	0.00 ± 0.00	9.00 ± 0.53	Evans and Cohen, 1989b
6-OH-DOPA[a] (n=12)	5.54 ± 0.42	9.40 ± 0.46	
Control (n=8)	0.00 ± 0.00	8.10 ± 0.25	Seiden and Vosmer, 1984
Methyl-amphetamine[b] (n=6)	0.24 ± 0.21	6.40 ± 0.84	

[a]Animals were pretreated with pargyline (100 mg/kg, 18 h). 6-OH-DOPA (200 mg/kg) was co-injected with carbidopa (50 mg/kg). Data are at 2.5 hours after 6-OH-DOPA.
[b]Animals received d-methylamphetamine hydrochloride (100 mg/kg). Data are at 2 hours after methylamphetamine.

Yet the higher amounts seen in the current experiments are not associated with known degenerative effects on striatal DA terminals. The relative resistance of striatal DA terminals to the confirmed presence of significant quantities of 6-OHDA after administration of 6-OH-DOPA remains an observation of strong interest.

Chapter 5: Modulation of the toxicity of 6-OH-DOPA: DA levels and ^{3}H-DA uptake.

5.1 Introduction

The known resistance of dopaminergic neurons to systemic administration of 6-OH-DOPA is especially interesting in light of the observation that the striatum, an area receiving a dense dopaminergic innervation, contains sizable quantities of 6-OHDA (Table 6). Experiments with reserpine (Fig. 5) indicate that striatal 6-OHDA is stored within dopaminergic nerve terminals. The following experiments examined the possibility of potentiating the neurotoxic effects of 6-OH-DOPA by interfering with known protective mechanisms in hopes of better understanding the means by which DA neurons protect themselves from seemingly large amounts of intracellular 6-OHDA.

Three independent mechanisms were explored. The first is based on the observation by Reinhard et al. (1988) that storage of MPP^{+} (1-methyl-4-phenylpyridinium ion) in vesicles is protective against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced toxicity. By preventing uptake and storage by vesicles with either reserpine or tetrabenazine, these workers were able to augment the decrease in DA levels seen after MPTP.

Alternate means of potentiation are based on the known mechanism of toxicity of 6-OHDA. Both 6-OHDA and 6-OH-DOPA are remarkably unstable at neutral pH in vitro and react

with oxygen to generate H_2O_2 (Heikkila and Cohen, 1972; Graham et al., 1978). In vivo, the quinoidal (oxidized) products of 6-OHDA are recycled by tissue ascorbate (Heikkila and Cohen, 1972). Redox cycling of 6-OHDA is a highly effective means of amplifying the formation of H_2O_2. H_2O_2 can be toxic by itself, and it can also react with iron in its reduced form (ferrous, Fe^{2+}) to produce the highly reactive and damaging hydroxyl radical ($^{\cdot}OH$). Therefore, the second approach (Section 5.3) involved the administration of iron to mice in order to elevate brain iron levels as a means of increasing the likelihood of the reaction of 6-OHDA-generated H_2O_2 with tissue iron stores.

Brain iron levels in mature rodents are known to be well conserved and thus are difficult to manipulate. Preweanling mice, however, have an incomplete blood-brain barrier and have been shown to be vulnerable to the effects of drugs that do not normally penetrate the central nervous system in adults such as L-buthionine sulfoximine (Calvin et al., 1986; Slivka et al., 1988). Preweanling mice received repeated injections of iron-dextran in order to examine the combined effects of elevated brain iron and 6-OH-DOPA on levels of endogenous catecholamines.

A third set of experiments was aimed at potentiating the toxicity of 6-OHDA by interfering with known protective mechanisms. In vivo, the enzyme glutathione peroxidase is the major means of detoxification of H_2O_2 and organic

peroxides. This is particularly true for for MAO-generated H_2O_2 (Oshino and Chance, 1977). Removal of glutathione compromises glutathione peroxidase as well as any direct protective effect of glutathione. Pileblad et al. (1989) have potentiated the toxic effects of 6-OHDA (i.c.v) by pretreating rats with L-buthionine sulfoximine (i.c.v.), an inhibitor of the enzyme catalyzing the first step in the biosynthesis of glutathione namely, gamma-glutamylcysteine synthetase. An alternate means of compromising glutathione is by treating animals with diethylmaleate (DEM, maleic acid diethyl ester) a compound known to decrease tissue glutathione levels by reacting with glutathione either directly or through a reaction catalyzed by glutathione S-transferase (Boyland and Chasseaud, 1970 and Plummer et al., 1981). In Section 5.4 mice are treated with DEM in order to test for a possible effect of decreased GSH on the uptake of ^{3}H-DA into striatal synaptosomes after 6-OH-DOPA. Although removal of glutathione should potentiate the neurotoxicity in both dopaminergic and noradrenergic terminals, it is expected that interference with a major protective mechanism would make the residual sensitivity of dopaminergic and noradrenergic terminals more comparable.

5.2 Blockade of vesicular storage

As seen in Fig. 5, reserpine blocks the storage of 6-OHDA in vesicles. Reinhard et al., (1988), provided evidence that storage of MPP^+ in vesicles was protective against MPTP-induced toxicity (as measured by decreases in DA levels): MPTP toxicity was greater in mice pretreated with agents known to block vesicular storage. Experiments were conducted to determine whether similar results could be obtained with 6-OH-DOPA.

Experiments were carried out in which mice were pretreated with pargyline (100 mg/kg i.p.) 2 hours prior to reserpine (5 mg/kg i.p.). Pargyline at this dose inhibits both MAO A and MAO B (Buu and Angers, 1987). 6-OH-DOPA (200 mg/kg i.p.) was co-injected with carbidopa (50 mg/kg) at 2 hours following reserpine. Striata were analyzed for uptake of ^{3}H-DA at 15 hours after injection of 6-OH-DOPA or vehicle (Table 10; Experiment I). Uptake of ^{3}H-DA into striatal synaptosomes from animals receiving both 6-OH-DOPA and reserpine was somewhat reduced as compared to mice treated with reserpine alone. However this decrease did not reach statistical significance. Subsequent attempts at repeating this experiment proved difficult due to a high mortality rate. The combination of a high dose of pargyline with reserpine led to behavioral abnormalities (i.e., stereotypies, high irritability) and was frequently lethal especially when combined with administration of 6-OH-DOPA.

Table 10. Effect of pretreatment with reserpine on the uptake of ^{3}H-DA into striatal synaptosomes of control and 6-OH-DOPA-treated mice.

Control	Reserpine	Reserpine + 6-OH-DOPA
	Uptake (cpm x 10^3 ± SEM)	
Experiment I. (n=7)		
28.5 ± 3.2	22.8 ± 2.1 (80.0%)	17.0 ± 4.4 (59.6%)
Experiment II. (n=8)		
30.2 ± 3.8	23.7 ± 3.4 (78.5%)	21.2 ± 3.0 (70.2%)

Uptake and storage of ^{3}H-DA was carried out for a 10 minute period. Values in parentheses are percent of control. Data are the observed radioactivity accumulated by 1.83 mg of original tissue. Animals were pretreated with either pargyline (100 mg/kg i.p., experiment I) or clorgyline (2.5 mg/kg i.p., experiment II) 2 hours prior to injection of reserpine (5 mg/kg i.p.) or vehicle. Where indicated 6-OH-DOPA (200 mg/kg i.p.) was co-injected with carbidopa (50 mg/kg). Tissues were analyzed at 15 hours after injection of 6-OH-DOPA or vehicle. Each experiment is actually the pooled result of 2 individual experiments.
Statistics were performed by single factor ANOVA
Experiment I. p=0.0776 across groups
Experiment II. p=0.1756 across groups

In order to reduce mortality, in subsequent experiments, mice were pretreated with clorgyline (2.5 mg/kg i.p.), instead of pargyline (Table 10; Experiment II). Clorgyline, at this dose, selectively inhibits MAO A (Johnston, 1968). Treatment with clorgyline, in place of pargyline, reduced the mortality rate to zero. In addition, animals receiving both reserpine and clorgyline did not show any of the behavioral abnormalities seen in animals treated with reserpine and pargyline. The mean value for uptake of ^{3}H-DA into striatal synaptosomes from animals receiving both reserpine and 6-OH-DOPA was only somewhat lower than the mean value for mice treated with reserpine alone. This apparent decrease did not achieve statistical significance.

In separate experiments, mice were pretreated with tetrabenazine (25 mg/kg i.p.), instead of reserpine. Reinhard et al. (1988) reported that this dose of tetrabenazine potentiates the toxic effects of MPTP in mice. Unlike the effects of reserpinization, which are irreversible and long-lasting, the effects of tetrabenazine are reversible and short-acting (Quinn et al., 1959). For this reason, 6-OH-DOPA (200 mg/kg i.p.) was co-injected with carbidopa (50 mg/kg) soon after (30 minutes) tetrabenazine administration. Pretreatment with tetrabenazine did not cause a reduction in the uptake of ^{3}H-DA into striatal synaptosomes in 6-OH-DOPA-treated animals (Table 11).

In the experiments with pargyline-pretreated animals (Table 10), 2 mice out of 7 showed markedly decreased uptake

of ^{3}H-DA after treatment with reserpine plus 6-OH-DOPA: Uptakes were only 16% and 26% of the mean value for mice treated with reserpine alone (compared with a low of 64% for mice treated with reserpine alone). While these results seemed encouraging, subsequent experiments with mice treated with clorgyline plus reserpine (Table 10) or with pargyline plus tetrabenazine (Table 11) indicated that interference with vesicular storage does not sensitize dopaminergic neurons to 6-OH-DOPA. The 2 sensitive animals in the pargyline plus reserpine group may have represented the combined toxic effects of reserpine plus pargyline, which, as noted above, induced behavioral abnormalities and death.

Table 11. Lack of effect of tetrabenazine on the uptake of ^{3}H-DA into striatal synaptosomes from control and 6-OH-DOPA-treated mice.

Control	TBZ	6-OH-DOPA	6-OH-DOPA + TBZ
	Uptake (cpm x 10^3 ± SEM)		
26.8 ± 3.1	32.9 ± 8.7	26.5 ± 3.8	40.6 ± 7.4

Uptake and storage of ^{3}H-DA was carried out for a 10 minute period. Data are the observed radioactivity accumulated by 1.83 mg of original tissue. All animals were pretreated with pargyline (100 mg/kg i.p.) 18 hours prior to injection of tetrabenazine (25 mg/kg i.p., TBZ) or vehicle. Where indicated 6-OH-DOPA (200 mg/kg i.p.) was co-injected with carbidopa (50 mg/kg) 30 minutes after tetrabenazine. Tissues were analyzed at 2.5 hours after 6-OH-DOPA.
Statistics were performed by single factor ANOVA.
p=0.3734 across groups, n=4 animals per group.

5.3 Increase of brain iron levels

In experiments conducted jointly with another graduate student, iron-dextran was administered to preweanling mice. The study of iron levels per se in brain is not part of this thesis. However, iron levels were elevated, as described below. This experimental paradigm permitted a test of the interaction of elevated brain iron with 6-OH-DOPA. Table 12 shows the effects of iron administration on the levels of NE, DA, 6-OH-DOPA, and 6-OHDA in striatum of control and 6-OH-DOPA-treated mice. Preweanling mice (16 days) received a total of 20 mg of iron-dextran over 8 days (2.5 mg/day s.c., 50 mg/ml). 6-OH-DOPA was co-injected with carbidopa 24 hours after the last iron injection. The levels of iron in striatum at this time were significantly elevated in iron-treated animals (from 6.83 $\pm$ 0.95 ug/g (SEM, n=9) to 57.52 ug/g (n=10) $p < 0.001$, Student t-test). Levels of DA were not significantly altered by iron, 6-OH-DOPA, or combined iron/6-OH-DOPA treatments. In contrast, striatal levels of NE in iron-treated animals were significantly greater than those of controls (Table 12). NE levels in preweanling mice receiving both iron and 6-OH-DOPA were significantly lower than those of animals receiving iron alone, whereas NE levels in 6-OH-DOPA-treated animals were not different from controls. While iron treatment had no effect on levels of 6-OH-DOPA, levels of 6-OHDA in iron-treated animals were only 49.5% of those of 6-OH-DOPA treated controls.

Table 12. Effect of iron-dextran injections on levels of NE, DA, 6-OH-DOPA, and 6-OHDA in striatum of control and 6-OH-DOPA-treated preweanling mice at 24 hours after cessation of iron dextran treatment.

	NE	DA	6-OH-DOPA	6-OHDA
	(ug/g ± SEM)			
Control (n=5)	0.18 ±0.01	7.84 ±0.75	0.00 ±0.00	0.00 ±0.00
Iron (n=5)	0.26[a] ±0.01	8.91 ±0.92	0.00 ±0.00	0.00 ±0.00
6-OH-DOPA (n=5)	0.18[b] ±0.00	7.48 ±0.41	0.48 ±0.05	4.99 ±0.53
Iron + 6-OH-DOPA (n=4)	0.20[b] ±0.02	8.68 ±0.40	0.56 +0.05	2.47[c] ±0.74

16 day old mice received a total of 20 mg of iron dextran (2.5 mg/day s.c.) over 8 days. 6-OH-DOPA (200 mg/kg i.p., 2.5 hours) and carbidopa (50 mg/kg, i.p.) were co-injected 24 hours after the last iron injection. All animals received pargyline (100 mg/kg,i.p.) 18 hours prior to 6-OH-DOPA administration. Statistics were performed by single factor ANOVA followed by Fisher's PLSD.
NE $p=0.0024$ across groups
DA $p=0.4203$ across groups
6-OH-DOPA $p=0.3462$ across groups
6-OHDA $p=0.0001$ across groups
[a] $p < 0.05$ as compared to control
[b] $p < 0.05$ as compared to iron alone
[c] $p < 0.05$ as compared to 6-OH-DOPA alone

The effect of increasing the latency period between cessation of iron injections and 6-OH-DOPA administration was explored in a separate experiments. Table 13 shows results at 1 week after cessation of iron treatment. Preweanling mice (14 days) received a total of 20 mg of iron over 7 days. 6-OH-DOPA (200 mg/kg with pargyline and carbidopa) was injected 1 week after the last iron injection. As determined separately, the levels of iron in striatum at this time were still elevated as compared to control animals (from 15.06 ug/g (SEM, n=10) to 31.15 ug/g (n=12), $p < 0.001$ Student t-test).

Table 13 shows levels of NE, DA, and 6-OHDA in striatum of mice at 2.5 hour after 6-OH-DOPA. In accord with Table 12 (24 hours after cessation of iron treatment), iron treatment did not significantly alter DA levels either alone or in combination with 6-OH-DOPA. However, once again, levels of NE were significantly greater in iron treated mice as compared to controls. In addition, NE levels in mice receiving both iron and 6-OH-DOPA were significantly lower than mice receiving iron alone; similarly, NE levels in 6-OH-DOPA treated mice were decreased as compared to their respective controls. In contrast to the results in Table 12 (24 hours after the last iron injection), 6-OHDA levels in animals receiving both iron and 6-OH-DOPA were similar to those of animals receiving 6-OH-DOPA alone.

Table 13. Effect of iron-dextran injections on levels of NE, DA, 6-OH-DOPA, and 6-OHDA in striatum of control and 6-OH-DOPA-treated preweanling mice at 1 week after cessation of iron treatments.

	NE	DA	6-OHDA
		(ug/g ± SEM)	
Control (n=5)	0.19 ± 0.02	7.42 ± 0.44	0.00 ± 0.00
Iron (n=5)	0.28 ± 0.02[a]	7.43 ± 0.51	0.00 ± 0.00
6-OH-DOPA (n=4)	0.12 ± 0.01[ab*] *(n=3)	6.90 ± 0.09	5.93 ± 0.67
Iron + 6-OH-DOPA (n=5)	0.14 ± 0.01[ab]	8.02 ± 0.55	5.65 ± 0.37

14 day old mice received a total of 20 mg of iron dextran over 7 days. 6-OH-DOPA (200 mg/kg i.p., 2.5 hours) and carbidopa (50 mg/kg i.p.) were coinjected at 1 week after the last iron injection. All animals were pretreated with pargyline (100 mg/kg i.p.) 18 hours prior to 6-OH-DOPA or vehicle.
Statistics were performed by single factor ANOVA followed by Fisher's PLSD.
NE $p=0.0001$ across groups
DA $p=0.4490$ across groups
6-OHDA $p=0.7083$ across groups
[a] $p < 0.05$ as compared to control
[b] $p < 0.05$ as compared to iron alone

5.4 Interference with peroxide detoxification

5.4.1 Effect of pretreatment with diethyl maleate on the uptake of ^{3}H-DA in control and 6-OH-DOPA treated mice.

The alpha,beta-unsaturated carbonyl compound, diethylmaleate (DEM), depletes glutathione (GSH) from liver of rats, mice, and hamsters (Boyland and Chasseaud, 1967, 1970; Plummer et al., 1981). DEM also depletes GSH, although to a lesser extent, from erythrocytes, kidney, lung, and brain (Boyland and Chasseaud, 1967). Slivka et al. (1987) measured a 66% decrease in whole brain GSH levels 30 minutes after DEM (1 ml/kg, i.p.). DEM depletes GSH both directly and through a reaction catalyzed by glutathione S-transferase (Boyland and Chasseaud, 1970 and Plummer et al., 1981).

Mice were pretreated with DEM in order to reduce brain GSH, as a potential means of potentiating the toxic effects of 6-OH-DOPA. Levels of GSH in striatum of mice treated with DEM were reduced by 49% and 75% by 1 and 2 injections of DEM (1 ml/kg i.p., 30 minute interval, Table 14) respectively. However, uptake of ^{3}H-DA into striatal synaptosomes was not affected by DEM-induced reductions in tissue GSH levels, nor did DEM potentiate the effect of 6-OH-DOPA. (Table 15).

Table 14. Concentration of GSH in the striatum of control and DEM treated mice.

Treatment group	Concentration GSH (mM, mean ± SEM)	% Control
Control (n=9)	1.67 ± 0.08	
DEM (1 ml/kg) (n=8)	0.86 ± 0.18[a]	51.5 %
DEM (2 x 1 ml/kg) (n=5)	0.41 ± 0.13[a,b]	24.6 %

All animals were pretreated with pargyline (100 mg/kg i.p., 18 hours). Where indicated animals received 1 or 2 injections of DEM (1 ml/kg i.p., 30 minute interval). Levels of GSH in striatum were measured at 30 minutes.
Statistical analysis were performed by single factor ANOVA followed by Fisher's PLSD ($p=0.0001$ across groups).
[a] $p < 0.05$ as compared to control
[b] $p < 0.05$ as compared to DEM (1 ml/kg)

Table 15. Effect of pretreatment with DEM on the uptake of ^{3}H-DA into striatal synaptosomes of control and 6-OH-DOPA -treated mice.

	Uptake (cpm x 10^3 ± SEM)	% Control
Control (n=7)	21.4 ± 1.5	
DEM (n=7)	19.2 ± 0.6	89.7 %
DEM + 6-OH-DOPA (n=7)	18.9 ± 1.6	88.3 %

Uptake and storage of ^{3}H-DA was carried out for a 10 minute period. Data are the observed radioactivity accumulated by 1.83 mg of original tissue. DEM (2 x 1 ml/kg i.p.) was administered 60 and 30 minutes prior to co-injection of 6-OH-DOPA (200 mg/kg i.p., 2.5 hours) and carbidopa (50 mg/kg i.p.). All animals were pretreated with pargyline (100 mg/kg i.p., 18 hours).
Statistics were performed by single factor ANOVA ($p=0.3491$ across groups).

5.5 Discussion

The experiments in Section 5.2 explore the possibility that storage of 6-OHDA in vesicles might be protective. In an early study with mice, Jonsson and Sachs (1970) investigated NE levels in the heart 1 week after treatment with 6-OHDA (2 x 50 mg/kg i.v., 16 hour interval). Some mice were pretreated with reserpine (10 mg/kg i.p., 16 hours prior to injection of 6-OHDA). Treatment with 6-OHDA alone caused a 75% reduction in heart NE. Pretreatment with reserpine, prior to 6-OHDA, did not cause a further reduction in NE levels (77% reduction). Similarly, in the current study (Table 10), pretreatment with reserpine did not sensitize dopaminergic terminals of the striatum to 6-OH-DOPA.

Experiments with tetrabenazine were also performed. Tetrabenazine did not potentiate the effect of 6-OH-DOPA. Values for tetrabenazine-treated animals were actually somewhat higher (Table 11) than their respective controls, although this effect was also not statistically significant. These data indicate that pretreatment with tetrabenazine does not sensitize dopaminergic terminals to 6-OH-DOPA.

Clearly, storage in vesicles does not play a major role in the resistance of dopaminergic terminals to 6-OH-DOPA. However, the existence of a pronounced effect in a few animals treated with reserpine and 6-OH-DOPA (text) cannot be ignored, and may indicate that storage plays a limited

role in this process.

The concept that accumulations of iron might enhance lipid peroxidation in vivo has been suggested. Dexter et al. (1987, 1989a) and Sofic et al.(1988) have reported increased iron and malondialdehyde (Dexter et al., 1986, 1989b), an index of lipid peroxidation, in the substantia nigra of post-mortem parkinsonian brain. These workers proposed that the increased availability of iron in parkinsonian nigra might present an oxidative stress.

In support of this theory, Willmore and coworkers have have experimental evidence that solutions of iron salts placed directly into rat brain, will induce lipid peroxidation (Willmore et al., 1983; Willmore and Rubin, 1984; Triggs and Willmore, 1984; Willmore et al., 1986). Willmore et al. (1983) observed the formation of superoxide radicals in brain (as measured by nitroblue tetrazolium assay) at 5 and 15 minutes after injection of 5 uL of 100 mM $FeCl_3$ into rat isocortex. Similarly, Triggs and Willmore (1984) observed the appearance of fluorescent products of lipid peroxidation in chloroform-methanol extracts of isocortical homogenates 2 hours after injection of 10 uL of $FeCl_2$ into rat isocortex.

Iron in its reduced form (ferrous, Fe^{2+}) or iron chelated with ligands such as citrate, ATP, or ADP, can facilitate lipid peroxidation in part through a reaction with H_2O_2 to form the hydroxyl radical ($^{\cdot}OH$). In addition, iron can also catalyze the autoxidation of catecholamines

leading to the formation of superoxide (O_2^-) and H_2O_2 (Halliwell and Gutteridge, 1986).

In the current study (Tables 12 and 13), preweanling mice (14-16 days) were treated with iron dextran as a means of elevating brain iron levels. Oberhauser et al. (1970) had previously demonstrated that iron-overloading with iron dextran in mature rabbits and monkeys caused increased iron leves in the basal ganglia, but not in the cerebral cortex of iron dextran-treated animals. The experiments in Section 5.3 test for a possible potentiation of 6-OH-DOPA-induced toxicity through the interaction of H_2O_2 (formed through the autoxidation and redox cycling of 6-OHDA) and iron. As seen in Tables 12 and 13, iron-overloading does not sensitize dopaminergic neurons to 6-OH-DOPA at either 24 hours or 1 week after cessation of iron dextran injections. One possible explanation for a lack of sensitization at 24 hours after cessation of iron dextran injections, is the observed lower concentration of 6-OHDA in iron dextran-treated animals (Table 12). The lesser amount of 6-OHDA could not be explained by a lower amount of 6-OH-DOPA in iron dextran-treated animals in this experiment. The observed levels of 6-OHDA may indicate a possible iron-catalyzed autoxidation of 6-OHDA.

While iron dextran treatments clearly had no affect on DA levels in striatum, NE levels in striatum in iron dextran-treated animals were significantly increased as

compared to controls at both 24 hours and one week after cessation of iron dextran injections (Tables 12 and 13). In addition at 24 hours after iron dextran, the level of NE in animals receiving both iron dextran and 6-OH-DOPA was lower (Table 12) than the levels of NE in animals treated with iron alone, whereas NE levels in animals receiving 6-OH-DOPA alone were not different from their respective controls. Similarly, at one week after iron dextran (Table 13), NE levels in animals receiving iron dextran and 6-OH-DOPA were also reduced as compared to iron dextran-treated controls; however, in this experiment, NE levels in 6-OH-DOPA-treated animals were also reduced as compared to untreated controls. The observation that iron dextran elevates striatal NE levels at both time points is also of interest.

In Section 5.4 the possibility of sensitizing dopaminergic neurons to 6-OH-DOPA-induced toxicity by compromising known protective mechanisms was explored. In a recent study, Pileblad et al. (1989) showed that the toxic effects of 6-OHDA placed directly into the lateral ventricles could be potentiated in rats pretreated with L-buthionine sulfoximine, an inhibitor of gamma-glutamylcysteine synthetase, the enzyme catalyzing the first step in glutathione biosynthesis. Pretreatment with L-buthionine sulfoximine essentially doubled the efficacy of i.c.v. 6-OHDA: In the striatum, the loss of DA was increased from 14% to 31% and the loss of NE was increased from 34% to 69% with 150 ug of 6-OHDA, while with a higher dose of 300

ug 6-OHDA, the loss of DA was increased from 36% to 60%, and the loss of NE was increased from 62% to 74%.

In the current study an alternate means of depleting glutathione was explored. Diethyl maleate (maleic acid diethyl ester, DEM) depletes glutathione directly and through a reaction catalyzed by glutathione S-transferase. (Boyland and Chasseaud, 1970; Plummer et al., 1981). The effects of DEM on levels of GSH (reduced glutathione) in striatum were studied (Table 14). Slivka et al. (1987) had shown previously that injection of DEM (1 mL/kg, i.p.) decreased whole brain GSH levels in mice by 66%. In the current study, a single injection of DEM (1 mL/kg i.p.) decreased striatal GSH by 49%, while 2 injections (1 mL/kg i.p., 30 minute interval) decreased striatal GSH by 75% (Table 14). However, in subsequent experiments (Table 15), pretreatment with 2 (1 mL/kg i.p., 30 minute interval) injections of DEM did not sensitize dopaminergic neurons to 6-OH-DOPA induced toxicity.

One possible explanation for a lack of potentiation by DEM is that DEM might have depleted the glia pool of GSH, but not the neuronal pool. As mentioned previously, DEM depletes GSH, at least in part, through a reaction catalyzed by glutathione S-transferase. Senjo et al. (1985), demonstrated that, antibodies staining for glutathione S-transferase stained astrocytes in either the cortex or the medulla of rat brain, as well as ependymal cells, and

choroid plexus epithelium in the ventricle, but not neurons or oligodendrocytes. Alternatively, high concentrations of 6-OHDA in dopaminergic terminals may completely deplete GSH levels, making it impossible to further deplete GSH with DEM.

In summation, these experiments rule out storage in vesicles as a reason for the differential sensitivities of dopaminergic and noradrenergic neurons to 6-OH-DOPA. Neither the experiments with iron-dextran loading nor the removal of GSH with DEM led to sensitization of dopaminergic neurons. Thus, the reason(s) for the remarkable difference between dopaminergic and noradrenergic neurons remains an unanswered question.

Chapter 6: Effects of reserpine-induced increases in DA metabolism

6.1 Introduction

Reserpine binds irreversibly to amine storage vesicles causing rapid depletions of central and peripheral monoamines (Carlsson et al., 1957). The mechanism of action of reserpine involves inhibition of Mg^{++}-ATP dependent uptake of amines into storage vesicles (Carlsson, Hillarp, and Waldeck, 1963; Dahlstrom, Fuxe, and Hillarp, 1965). The effects on levels of endogenous amines are surprisingly long-lasting. At 3 weeks after a single injection of reserpine (5 mg/kg i.p.), levels of DA, NE, epinephrine, and serotonin in the striatum, limbic region, hippocampus, and hypothalamus of rats are still below control values (Ponzio et al., 1984; Algeri et al., 1987). While the reappearance of endogenous amines is known to involve the synthesis of new vesicles, it is not clear that this is the only factor involved. For example, the ability to take up and store intracerebroventricularly administered ^{3}H-NE is severely compromised at 6 hours after a single injection of reserpine (2 mg/kg i.p.), recovers rapidly between 24-48 hours after reserpinization, and is almost normal by 8 days; however, at 8 days, NE levels are only 40% of control values (Glowinski, Iversen, and Axelrod, 1966). Return of the ability to accumulate ^{3}H-NE centrally parallels the reestablishment of functionality in peripheral

sympathetic nerves (Anden, Magnusson, and Waldeck, 1964).

The experiments that follow explore various aspects of reserpine-induced changes in striatal DA levels in mice. These measurements include levels of DA and metabolites, uptake, storage, number of uptake sites (as reflected by ^{3}H-mazindol binding), and tyrosine hydroxylase activity. One reason for examining the long-lasting action of reserpine more closely is that recent work has shown that elevations in H_2O_2, resulting from reserpine-induced increases in DA turnover, are associated with an "oxidative stress", as indicated by an accumulation of glutathione disulfide (Spina and Cohen, 1989). Oxidized glutathione (GSSG) is produced through the detoxification of H_2O_2 by glutathione peroxidase. Elevated levels of GSSG and/or H_2O_2 could conceivably lead to metabolically damaging events either through the formation of mixed disulfides or through the oxidation of sulfhydryl-dependent enzymes as discussed in Section 6.7.

6.2 Levels of DA and metabolites in mouse striatum after reserpinization

As a first step, recent reports (Ponzio et al., 1984; Algeri et al., 1987) that DA levels remain decreased in the striatum for several weeks following reserpine administration were confirmed. Tables 16 and 17 show the short- and long-term effects in mice of a single injection of reserpine (10 mg/kg i.p.). As seen in Table 16 (short-term effects), striatal DA levels were reduced by 98% and 95%, respectively, at 5 and 24 hours after reserpine. DA levels rose gradually over the next 24 hours, but remained reduced by 75% at the 48 hour time point. The marked initial fall in striatal DA was accompanied by a pronounced increase in DOPAC and HVA, reflecting loss of vesicular DA and increased turnover of DA by MAO. At 5 and 24 hours after reserpine, levels of DOPAC had increased by 2.6-fold and 2.3-fold, respectively. Levels of DOPAC at 48 hours were still 1.8-fold greater than control levels. Similarly, HVA levels at 5 and 24 hours after reserpine were 2.0-fold those of controls. This rise in metabolites reflects increased metabolism of DA by MAO; the results are consistent with the literature on reserpine.

Table 16. Short-term effects of pretreatment with reserpine on levels of DA and metabolites in mouse striatum.

	DA	DOPAC	HVA
	(ug/g mean ± SEM)		
Controls (n=5)	7.66 ±0.61	0.69 ±0.07	1.15 ±0.10
5 hours (n=5)	0.15[a] ±0.01	1.78[a] ±0.23	2.27[a] ±0.21
24 hours (n=5)	0.39[a] ±0.08	1.56[a] ±0.15	2.26[a] ±0.11
48 hours (n=5)	1.94[a] ±0.76	1.22[a] ±0.20	1.52 ±0.18

Animals were treated with reserpine (10 mg/kg, i.p.) or vehicle.
Statistics were performed by single factor ANOVA followed by Fisher's PLSD ($p=0.0001$ across groups).
[a] $p < 0.05$ as compared to control

Table 17. Long-term effects of pretreatment with reserpine on levels of DA and metabolites in mouse striatum.

	DA	DOPAC	HVA
	(ug/g mean ± SEM)		
Controls (n=5)	8.57 ±0.70	0.68 ±0.04	1.07 ±0.04
1 week (n=5)	2.86[a] ±0.09	0.47[a] ±0.03	0.70[a] ±0.04
2 weeks (n=5)	5.28[a] ±0.30	0.69 ±0.04	0.98 ±0.07
3 weeks (n=5)	6.99[a] ±0.20	0.75 ±0.04	1.09 ±0.09

Animals were treated with reserpine (10 mg/kg, i.p.) or vehicle.
Statistics were performed by single factor ANOVA followed by Fisher's PLSD ($p=0.0001$ across groups).
[a] $p < 0.05$ as compared to control

DA levels continued to rise slowly after 48 hours (Table 17). At one week, DA levels in reserpinized animals were only 33% of controls. At 2 and 3 weeks, DA levels remained significantly reduced at 62% and 82% of controls respectively. These results confirm the findings of Ponzio et al. (1984) and Algeri et al. (1987) in which levels of striatal DA in rats were initially depleted and then gradually increased, but had not returned to normal at 3 weeks after a single injection (5 mg/kg i.p.) of reserpine. Striatal DOPAC and HVA had returned to normal by 2 weeks; however lowered DA levels were present for 3 weeks or longer.

6.3 Effect of MAO inhibition by clorgyline on the recovery of DA levels after reserpinization

Experiments were designed to explore the effects of reserpine-induced increases in MAO activity on the slow reappearance of DA after reserpinization. The effect of inhibiting MAO (to prevent formation of H_2O_2) was studied in control and reserpinized animals (Table 18). Animals were injected with clorgyline (2.5 mg/kg i.p.) or saline 18 hours prior to reserpine (10 mg/kg i.p.) or vehicle. Clorgyline at this dose selectively inhibits MAO A (Johnston, 1968). Clorgyline injections were continued at 48 hour intervals. Striata were analyzed for levels of DA, DOPAC, and HVA at 1 and 2 weeks after reserpine.

DA levels in clorgyline-treated control animals were increased slightly (1.41 ug/g at 1 week, 0.68 ug/g at 2 weeks) as compared to untreated controls (Table 18). This effect reached statistical significance at 1 week, but not at 2 weeks. As seen in Tables 17 and 18, DA levels at 1 and 2 weeks after reserpine, respectively, were 33-37% and 59-62% of controls. DA levels in animals receiving clorgyline plus reserpine were 2.05 and 2.00 ug/g greater at 1 and 2

Table 18. Effects of chronic clorgyline treatment on the levels of DA and metabolites in striatum of control and reserpine-treated mice.

		DA	DOPAC	HVA
			(ug/g ± SEM)	
1 WEEK				
CNTRL	(n=10)	9.51 ± 0.40	0.86 ± 0.05	1.03 ± 0.04
CLG	(n=10)	10.91 ± 0.56^{ab}	0.20 ± 0.01^{ab}	0.45 ± 0.02^{a}
RES	(n=10)	3.56 ± 0.15^{a}	0.63 ± 0.03^{a}	0.71 ± 0.02^{a}
RESCLG	(n=10)	5.61 ± 0.57^{ab}	0.14 ± 0.00^{ab}	0.29 ± 0.02^{ab}
2 WEEKS				
CNTRL	(n=9)	11.60 ± 0.40	0.88 ± 0.04	1.30 ± 0.09
CLG	(n=10)	12.28 ± 0.80^{b}	0.19 ± 0.02^{ab}	0.47 ± 0.03^{a}
RES	(n=10)	6.87 ± 0.34^{a}	0.72 ± 0.03^{a}	1.00 ± 0.08^{a}
RESCLG	(n=10)	8.87 ± 0.25^{ab}	0.14 ± 0.01^{ab}	0.40 ± 0.02^{ab}

Animals were pretreated with clorgyline (2.5 mg/kg i.p.) or saline 18 hours prior to a single injection of reserpine (10 mg/kg, i.p.) or vehicle. Clorgyline treated mice continued to receive clorgyline (2.5 mg/kg) at 48 hour intervals. Striatal levels of DA and metabolites were analyzed at 1 or 2 weeks following reserpinization. Results are pooled from 2 independent experiments at each time point.
Statistics were performed by single factor analysis of variance followed by Fisher's PLSD ($p=0.0001$ across groups).
[a] $p < 0.05$ as compared to control
[b] $p < 0.05$ as compared to reserpine

weeks, respectively, than those of corresponding animals receiving reserpine alone. These data show that clorgyline can enhance the recovery of DA. The results suggest that events associated with increased turnover of DA by MAO can interfere with the reappearance of DA levels after reserpinization. However the possibility that clorgyline can directly elevate DA levels in reserpine-treated animals is not excluded by these data and is addressed in separate experiments.

To rule out the possibility that clorgyline might have a direct effect on DA levels in reserpine-treated animals, experiments were performed in which reserpinized animals received clorgyline at the end of the experiment, prior to conducting assays for striatal DA. In this way, animals exposed to reserpine alone were provided the potential benefit of inhibition of MAO. In these experiments, animals received a single injection of reserpine (10 mg/kg i.p.). As described previously (Table 18), some animals were pretreated with clorgyline (2.5 mg/kg) prior to reserpinization and continued to receive clorgyline at 48 hour intervals. However, in order to normalize for inhibition of MAO, all animals received 2 injections of clorgyline (2.5 mg/kg i.p.) at 24 and 16 hours prior to analysis. As seen in Table 19, after normalization for inhibition of MAO, DA levels in animals protected by clorgyline during the reserpinization period were 1.39 ug/g higher than DA levels in reserpinized animals that received

Table 19. DA levels in striatum after normalization for inhibition of MAO after 1 week of reserpinization with and without chronic clorgyline treatment

	DA (ug/g ± SEM)
Reserpine week 1 (n=14)	6.17 ± 0.25
Res./Clg. week 1 (n=15)	7.56 ± 0.58[a]

Animals were pretreated with clorgyline (2.5 mg/kg i.p.) or saline 18 hours prior to a single injection of reserpine (10 mg/kg i.p.). Clorgyline-treated animals continued to receive clorgyline (2.5 mg/kg) at 48 hour intervals. All animals were injected with clorgyline (2.5 mg/kg) at 24 hours and 16 hours prior to assay. Striatal levels of DA were measured at 1 week after reserpinization. Results are pooled from 2 independent experiments.

[a] $p < 0.05$ as compared to reserpine alone (Student t-test)

clorgyline at the end of the experiment only. Although clorgyline may contribute to the elevation in DA levels in part by a direct action, these data suggest that clorgyline also allows for higher DA levels by preventing events associated with increased DA turnover.

Administration of clorgyline lowered levels of DOPAC more severly than HVA (Table 18). This observation is consistent with DOPAC representing an intraneuronal (presynaptic) metabolite of DA, and with the presence of MAO A in dopaminergic nerve terminals. Changes in DOPAC levels often serve as a useful index for presynaptic changes in MAO activity. HVA, in contrast, is generated by the extraneuronal enzyme, catechol O-methyltransferase, which acts directly on DOPAC as well as on DA that has been released into the synaptic cleft. O-Methylated DA can in turn be oxidatively deaminated by MAO B to generate HVA. Therefore, HVA is less affected than DOPAC after inhibition of MAO A by clorgyline.

6.4 Effect of pretreatment with reserpine or reserpine plus clorgyline on the uptake and storage of ^{3}H-DA and ^{3}H-mazindol binding.

Reserpine inhibits the uptake of monoamines into amine-storage vesicles. In performing "uptake" measurements, both transport across the axonal membrane and subsequent transport across the vesicular membrane can play a role, depending upon the conditions chosen. In general, short incubation times with ^{3}H-amine favor a study of the axonal membrane pump ("uptake"), while longer incubation times permit the monoamine vesicles ("storage") to play a role. As shown in Table 20, at one week after a single injection of reserpine, uptake into dopaminergic terminals, as defined by a one minute accumulation of ^{3}H-DA into striatal synaptosomes, was unaltered. Storage, as defined by a 10 minute accumulation, was decreased by 23.2% at one week after reserpine (5 mg/kg i.p.), but had returned to normal by 2 weeks after a 10 mg/kg (i.p.) dose of reserpine. In contrast to the results in Tables 18 and 19 (reserpine-induced changes in DA levels), chronic inhibition of MAO by clorgyline (as described previously) did not have a protective effect on the reserpine-induced decrease in storage at one week.

In contrast to synaptosomal uptake of ^{3}H-DA which is an index of the functional integrity of the terminal membrane, binding of ^{3}H-mazindol to striatal membranes is an index of

Table 20. Effect of pretreatment with reserpine or reserpine plus clorgyline on the uptake and storage of ^{3}H-DA in striatal synaptosomes.

	Control	Reserpine	Reserpine/Clorgyline
	(cpm x 10^3 ± SEM)		
1 week after reserpine (5 mg/kg)			
Uptake (n=6)	9.1 ± 1.4	8.9 ± 1.4 (97.5%)	7.9 ± 0.6(86.3%)
Storage (n=5)	35.4 ± 2.5	27.2 ± 2.4 (76.8%)[a]	22.1 ± 2.0(62.5%)[a]
2 weeks after reserpine (10 mg/kg)*			
Storage (n=8)	26.5 ± 2.3	26.6 ± 2.7 (100.5%)	25.7 ± 2.3(96.9%)

Data are the observed radioactivity accumulated by 1.83 mg of original tissue. Values in parentheses are percent of control uptake. All animals received reserpine (5 or 10 mg/kg i.p.) or saline. Where indicated animals were pretreated with clorgyline (2.5 mg/kg i.p., 18 hours) and continued to receive the same dose at 48 hour intervals. Uptake (1 minute) or storage (10 minutes) was assessed at 1 or 2 weeks after reserpinization.

*All animals received pargyline (100 mg/kg i.p. x 3 at 72 hour intervals prior to assay).

Statistics were performed by single factor ANOVA followed by Fisher's PLSD

uptake, p=0.7326 across groups

storage 1 week, p=0.0056 across groups

storage 2 weeks, p=0.9554 across groups

[a] $p < 0.05$ as compared to control

the structural integrity of the terminals. Mazindol is a catecholamine uptake blocker and therefore ^{3}H-mazindol binding labels the uptake sites on dopaminergic terminals (Javitch et al., 1984). A decrease in mazindol binding is used as an index of loss of terminals (e.g., Sershen et al., 1986). As shown in Table 21, binding of ^{3}H-mazindol to striatal membranes was unchanged at one week after reserpine (10 mg/kg i.p.)

A lack of effect on uptake or ^{3}H-mazindol binding at one week, as well as the return of DA storage to normal at 2 weeks after reserpinization, argues against the occurrence of overt toxicity or severe metabolic damage (such as loss of ATP) as an explanation for the persistent decreases in DA (Table 17) at one week (-67%) and at 2 weeks (-38%) after reserpinization.

Table 21. ^{3}H-Mazindol binding on striatal membranes from control and reserpine-treated mice.

Control	Reserpine
(cpm x 10^3 ± SEM)	
5.1 ± 0.7 (n=5)	4.6 ± 0.7 (n=5)

Data are the observed radioactivity bound to 2.47 mg of original tissue. Striata were analyzed for ^{3}H-mazindol binding at 1 week after reserpine (10 mg/kg i.p.) or vehicle. Data are pooled from 2 independent experiments. ($p > 0.60$ as compared to control, Student t-test)

6.5 Effect of pretreatment with reserpine or reserpine plus clorgyline on tyrosine hydroxylase activity in striatum.

Levels of monoamines within catecholamine neurons can be affected by tyrosine hydroxylase activity. Tyrosine hydroxylase can be measured in vitro in broken cell preparations by adding all necessary cofactors and protective agents (such as mercaptoethanol), or it can be measured in vivo in rodents by inhibiting DOPA-decarboxylase and studying the accumulation of L-DOPA. In the experiments that follow, the in vivo assay was used to assess the level of tyrosine hydroxylase activity as it exists within the environment of dopaminergic nerve terminals in the striatum.

As seen in Table 22, tyrosine hydroxylase activity, as measured by accumulation of L-DOPA at 30 minutes after administration of the centrally acting DOPA-decarboxylase inhibitor NSD-1015 (100 mg/kg, s.c.), was decreased by 10.9% at 1 week after reserpine (7.5 mg/kg i.p.). MAO inhibition by clorgyline, by itself, caused a 44.5% reduction in tyrosine hydroxylase activity which was significantly augmented in animals receiving both reserpine and clorgyline (60.9% decrease). The data with clorgyline alone confirm the in vitro observations of Schoepp and Azzaro (1981), who showed that inhibition of MAO-A, induced by incubating rat striatal slices with clorgyline, leads to decreased DA biosynthesis. The data with reserpine alone indicate that a depression of tyrosine hydroxylase activity is a long-range

Table 22. Effect of pretreatment with reserpine or reserpine plus clorgyline on tyrosine hydroxylase activity in striatum.

	L-DOPA ACCUMULATION	DA	HVA
		(ug/g ± SEM)	
Control (n=17)	1.28 ± 0.05	8.92 ± 0.22	0.84 ± 0.02
Clorgyline (n=5)	0.71 ± 0.06[a]	10.65 ± 0.72[a]	0.37 ± 0.03
Reserpine (n=16)	1.14 ± 0.05[a]	2.71 ± 0.16[a]	0.56 ± 0.02[a]
Res./Clg. (n=11)	0.50 ± 0.02[abc]	5.78 ± 0.35[abc]	0.29 ± 0.04[ab]

Animals received a single injection of reserpine (7.5 mg/kg i.p.) or saline. Some animals were pretreated with clorgyline (2.5 mg/kg i.p., 18 hours) and continued to receive the same dose at 48 hour intervals. At 1 week after reserpinization all animals were pretreated with NSD-1015 (100 mg/kg, s.c.). Tyrosine hydroxylase activity is expressed as accumulation of L-DOPA at 30 minutes after NSD-1015. Results are pooled from 2 independent experiments. Statistics were performed by single factor ANOVA followed by the Fisher's PLSD ($p=0.0001$ across groups).

[a] $p < 0.05$ as compared to control
[b] $p < 0.05$ as compared to reserpine alone
[c] $p < 0.05$ as compared to clorgyline alone

consequence of exposure to reserpine. However, assessment of a potential protective role of clorgyline is complicated by a direct action of clorgyline on tyrosine hydroxylase activity. The direct action of clorgyline probably reflects end-product inhibition of the enzyme by accumulation of DA in the cytosol (Schoepp and Azzaro, 1981). In the case of animals treated with reserpine plus clorgyline, this effect may be enhanced by a decrease in impulse flow mediated by receptor-activated feedback inhibition (Studer and Schultz, 1987).

6.6 Effect of chronic L-DOPA on the reappearance of DA levels after reserpine

As seen in Table 18, DA levels at 1 and 2 weeks after reserpine were greater in animals treated with both reserpine and clorgyline than in animals treated with reserpine alone. This effect persisted even when all animals received 2 injections of clorgyline prior to analysis in order to normalize for inhibition of MAO (Table 19). These data implicate elevated turnover by MAO as a mediating factor in the reappearance of striatal DA after reserpine. In order to further explore the effect of MAO, some animals were injected with L-DOPA (100 mg/kg i.p. with carbidopa) 24 hours after reserpine (5 mg/kg i.p.) as a means of enhancing reserpine-induced increases in DA turnover by providing additional substrate for MAO. L-DOPA injections were repeated at 24 hour intervals for 7 days. Analysis was preceded by a 7 day "wash-out" period in order to avoid a direct effect of L-DOPA on measure levels of DA. As seen in Table 23, DA levels in L-DOPA-treated animals were not further suppressed and were actually 11% higher than those of animals receiving reserpine alone. These data indicate that the effects seen with reserpine cannot be augmented further with L-DOPA.

Table 23. Effect of chronic L-DOPA on the levels of DA and metabolites after reserpine

	DA	DOPAC (ug/g ± SEM)	HVA
Reserpine (n=7)	5.50 ± 0.18	0.54 ± 0.01	0.80 ± 0.07
Reserpine + L-DOPA (n=10)	6.12 ± 0.20[a]	0.69 ± 0.02[b]	0.83 ± 0.02

All animals received a single injection of reserpine (5 mg/kg i.p.). L-DOPA (50 mg/kg i.p.) was co-injected with carbidopa (12.5 mg/kg) daily for 7 days starting 1 day after reserpinization. Striata were analyzed 7 days following the last L-DOPA injection.

[a] $p < 0.05$ as compared to reserpine alone

[b] $p < 0.001$ as compared to reserpine alone

(Student t-test)

6.7 Discussion

The long-lasting effects of reserpinization on levels of central and peripheral monoamines have been well documented (Carlsson et al., 1957; Dahlstrom and Haggendal, 1966; Haggendal and Dahlstrom, 1971; Ponzio et al., 1984; Algeri et al., 1987). The data in Tables 16 and 17 confirm the results of the latter studies and provide detailed results for levels of DA and its metabolites, DOPAC and HVA, at various times between 5 hours and 3 weeks. As shown in Table 16, at 5 hours after reserpine, the concentration of DA fell to 2% of control levels, while the concentrations of DOPAC and HVA were increased by 2.6-fold and 2.0-fold, respectively.

H_2O_2 is a major product of the oxidative deamination of DA by MAO (equation 1):

$$DA + O_2 + H_2O \xrightarrow{MAO} H_2O_2 + NH_3 + \text{3,4-dihydroxyphenylacetaldehyde} \quad (1)$$

Therefore, the sizable increases in DA metabolites observed after reserpine are accompanied by a considerable increase in production of H_2O_2.

H_2O_2, generated via the normal metabolism of DA by MAO, is detoxified via the glutathione peroxidase/glutathione reductase system (Oshino and Chance, 1977; Maker et al., 1981). In a reaction catalyzed by glutathione peroxidase, one molecule of H_2O_2 reacts with 2 molecules of reduced glutathione (GSH) to generate one molecule of oxidized

glutathione (GSSG) and 2 molecules of H_2O (equation 2). GSSG is then returned to its reduced form through the action of the NADPH-dependent GSSG reductase (equation 3).

$$H_2O_2 + 2GSH \xrightarrow{\text{GSH peroxidase}} GSSG + 2H_2O \quad (2)$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} + 2GSH + NADP^+ \quad (3)$$

Spina and Cohen (1989) observed an 87.8% increase in the level of oxidized glutathione (GSSG) in the striatum of mice at 2 hours after reserpine (10 mg/kg). Increases in GSSG were accompanied by a 97% decrease in DA levels and a 2-fold increase in levels of DOPAC and HVA. Pretreatment with the selective MAO-A inhibitor, clorgyline (2.5 mg/kg i.p., 18 hours), suppressed the reserpine-induced increases in GSSG by 88%. Similarly, the DA receptor antagonist haloperidol (1 mg/kg i.p., 1 hour) induced a 3-fold increase in striatal GSSG that was suppressed 72% by the selective MAO-B inhibitor, deprenyl (2.5 mg/kg i.p., 18 hours) (Cohen and Spina, 1989).

Elevations in striatal GSSG observed after reserpine or haloperidol imply that cellular detoxification mechanisms cannot accommodate the larger amounts of H_2O_2 generated by increased DA turnover. Spina and Cohen (1989) have suggested that the accumulation of GSSG after reserpine might result in changes in cellular components, including sulfhydryl-dependent enzymes and structural proteins, through the formation of mixed disulfides. Similar

suggestions have been made by other investigators for the effects of H_2O_2 in other tissues (Offerman et al., 1984; Bregelius, 1985). GSSG may also alter cellular activity by functioning as a "third-messenger" (Gilbert, 1982). In addition, loss of GSH can permit the direct oxidation of cellular constituents, such as lipids, by H_2O_2.

Deupree and Hitchcock (1988) described the inhibition of both ^{3}H-reserpine binding to and transport of ^{3}H-NE into chromaffin granule ghosts by micromolar amounts of the sulfhydryl reagent N-ethylmaleimide. Both changes could be prevented by preincubation with Mg^{++} and ATP. The authors suggested that the sulfhydryl group affected by N-ethylmaleimide may be localized to the proton translocator rather than the catecholamine transporter and that a membrane potential is required for both ^{3}H-reserpine binding and ^{3}H-NE uptake (Deupree and Hitchcock, 1988). These observations provide an example of how changes in the redox status of the cell can lead to alterations in metabolic events.

In order to determine whether the slow (3 weeks or longer) reappearance of DA levels after reserpine might be dependent upon reserpine-induced changes in redox status brought about by MAO activity, mice were pretreated with clorgyline (2.5 mg/kg i.p., 18 hours) prior to reserpinization, and clorgyline was readministered at 48 hour intervals. In this way, the indirect effects of MAO

on restoration of DA levels could be evaluated. Levels of DA and metabolites in striatum were assessed at 1 and 2 weeks. DA levels in animals receiving both reserpine and clorgyline were significantly greater than those of animals receiving reserpine alone (Table 18). The possibility of a simple, direct effect of clorgyline on DA levels in reserpine-treated animals was tested by injecting both reserpine-treated and reserpine/clorgyline-treated animals with 2 injections of clorgyline at 24 and 16 hours prior to analysis. In this way, MAO inhibition was normalized for both groups. The persistence of elevated DA levels in reserpine/clorgyline-treated animals after normalization for inhibition of MAO (Table 19) suggests that events linked to the metabolism of DA by MAO play a role in the slow rate of reappearance of DA levels after reserpine.

Analysis of uptake and storage of ^{3}H-DA by striatal synaptosomes indicates that the consequences of increased DA turnover do not include overt toxicity, such as loss of nerve terminals, per se, or damage to the membrane uptake mechanism. As seen in Table 20, at 1 week after reserpine (5 mg/kg i.p.), uptake was normal, while storage remained somewhat compromised. However, by 2 weeks after reserpine (10 mg/kg i.p.) storage had returned to normal as well. At the same time point, DA levels were at only 62% of control (Table 17).

A lack of an effect of reserpine on uptake of ^{3}H-DA into striatal synaptosomes at one week is consistent with a

lack of effect of reserpine on the binding of ^{3}H-mazindol to striatal membranes at the same time (Table 21). Mazindol binds to the reuptake site on catecholamine terminals. Therefore ^{3}H-mazindol binding can serve as a measure of the structural integrity of the the terminal, and a decrease in ^{3}H-mazindol binding is frequently used as an index of toxicity.

In separate experiments, tyrosine hydroxylase activity was assessed as an additional index of the vitality of DA nerve terminals. Tyrosine hydroxylase is the first and the rate limiting enzyme in the biosynthesis of catecholamines. A recent study (Grima et al., 1985) showed that tyrosine hydroxylase possesses 7 cysteine residues. It is known that changes in tyrosine hydroxylase activity can affect levels of DA in the striatum. For example, when haloperidol is administered, DA turnover is sharply increased, but DA levels rise; the rise in DA is associated with increased activity of tyrosine hydroxylase. Therefore, it is reasonable to expect that changes in DA levels in either an upward direction (e.g., haloperidol) or a downward direction (e.g., the long-term effect of reserpine) can reflect changes in tyrosine hydroxylase activity.

Tyrosine hydroxylase activity in vivo was assessed by the accumulation of L-DOPA at 30 minutes after administration of the centrally-acting DOPA-decarboxylase inhibitor (NSD-1015, 3-hydroxybenzylhydrazine

dihydrochloride, 100 mg/kg s.c.) (Nissbrandt and Carlsson, 1987; Nissbrandt et al., 1989). As seen in Table 22, tyrosine hydroxylase activity was reduced by 10.9% ($p < 0.05$) at one week after reserpine (7.5 mg/kg i.p.). Other workers have studied changes in tyrosine hydroxylase activity and tyrosine hydroxylase mRNA levels in different areas of rat brain after reserpine. Reis et al. (1975) observed a 341% increase in tyrosine hydroxylase activity in the locus ceruleus at 24 hours after reserpine treatment (4 x 2 mg/kg i.p., 24 hour intervals); however tyrosine hydroxylase activity was increased by only 20% in the hypothalamus and not at all in the substantia nigra or the striatum. Immunotitration with antibodies specific to tyrosine hydroxylase indicated that increased tyrosine hydroxylase activity in the locus ceruleus was due solely to the production of new enzyme protein (Reis et al., 1975). Similarly, Faucon Biguet et al. (1986) reported a 4.1 and a 4.5-fold increase in tyrosine hydroxylase mRNA in the adrenals and the locus ceruleus respectively, after a single injection of reserpine (10 mg/kg s.c.); increased mRNA was accompanied by an approximate doubling of tyrosine hydroxylase activity. These data suggest that increased tyrosine hydroxylase activity is the result of increased gene transcription. These workers failed to detect a change in either tyrosine hydroxylase activity or tyrosine hydroxylase message in the substantia nigra.

Finally, Labutat et al. (1988) studied both tyrosine

hydroxylase activity (by in vitro assay) and tyrosine hydroxylase protein levels (by immunoblot assay) in reserpine-treated and control animals. These workers observed a 2.7-fold increase in tyrosine hydroxylase activity that was paralleled by a 2.6-fold increase in tyrosine hydroxylase protein levels in the locus ceruleus at 4 days after reserpine (10 mg/kg s.c.), at which point both parameters had reached their maximal changes. In agreement with previous workers, reserpine failed to elicit an increase in tyrosine hydroxylase activity measured by in vitro assay in the substantia nigra; however, there was a 1.5-fold increase in tyrosine hydroxylase protein level in the substantia nigra that was maximal at 4 days after reserpine. Therefore, the "specific activity" of tyrosine hydroxylase in the substantia nigra appeared to be suppressed.

It is not clear why newly synthesized tyrosine hydroxylase would be active in the locus ceruleus and not in the nigra (Labutat et al., 1988). This phenomenon may be related to the observed decrease in tyrosine hydroxylase activity at 1 week after reserpine (Table 22). An apparent absence of a decrease in tyrosine hydroxylase activity in the striatum in the older studies (Reis et al., 1975) may reflect either variations in assay procedures (in vitro measurements vs. in vivo assays) and/or interspecies differences (the current study involves mice whereas the

previous work has been done with rats). With regard to in vitro assays, these are generally conducted with high levels of reducing agents, such as 28 mM mercaptoethanol (Reis et al., 1975), which can obscure or reverse effects due to mixed disulfides or other oxidative changes.

It is tempting to speculate that the decrease in tyrosine hydroxylase activity observed in vivo after reserpinization (Table 22) may be linked to the formation of non-functional tyrosine hydroxylase protein (Labutat et al., 1988). Such changes might be postulated to occur via the formation of a mixed disulfide between one or more of the 7 cysteine residues on tyrosine hydroxylase and one or more molecules of GSSG. On the other hand, a pair of cysteine residues on tyrosine hydroxylase might be oxidized by H_2O_2 and form a disulfide bond within an individual molecule of tyrosine hydroxylase. Evidence for alterations in enzyme catalysis due to this type of a structural change has been provided by Matsumura and Matthews (1989) who demonstrated that enzyme activity could be controlled (i.e., turned "on" or "off") via the reduction or oxidation of a disulfide bond engineered into the active-site cleft of a bacteriophage T4 lysozyme. Alternatively, a mixed disulfide could be formed by the oxidation of cysteine residues on 2 different molecules of tyrosine hydroxylase.

Chronic MAO inhibition by clorgyline decreased tyrosine hydroxylase activity by 44.5%. This clorgyline-induced decrease in tyrosine hydroxylase activity was even more

pronounced in animals receiving both reserpine and clorgyline (60.9% decrease). Schoepp and Azzaro (1981) reported decreased tyrosine hydroxylase activity as measured within intact cells of striatal slices after incubation with clorgyline in vitro. Javoy et al. (1972) observed a decreased rate of formation of $^{3}H\text{-}H_2O$ (an index of tyrosine hydroxylase activity) after incubating striatal slices with excess amounts of DA (10^{-5} M). This effect was partially prevented by incubation with the DA uptake blocker benztropine (10^{-6}M). In addition, Javoy et al. (1972) related observed decreases in DA synthesis to increases in DA levels in vivo by measuring the rate of accumulation of radiolabeled DA and 3-O-methyl-DA from ^{3}H-tyrosine after non-selective inhibition of MAO by pargyline.

Decreased tyrosine hydroxylase activity in clorgyline-treated animals is probably due to feedback inhibition of tyrosine hydroxylase by newly synthesized DA via competitive inhibition of the enzyme by its endproduct (Javoy et al., 1972; Schoepp and Azzaro, 1981) and/or increased transmitter-receptor interaction. The latter could occur via a multineuronal feedback loop (Studer and Schultz, 1987) or at the dendritic level (Bjorklund and Lindvall, 1975; Nieoullon et al., 1977).

Increased neurotransmitter-receptor interaction would be expected to decrease the firing rate, which in turn, might down-regulate tyrosine hydroxylase. Murrin et al.

(1976) observed increases in tyrosine hydroxylase activity as determined by an in vitro assay system after electrical stimulation of nigrostriatal neurons. This increased activity was characterized by a decreased K_m (increased affinity) of tyrosine hydroxylase for its substrate (tyrosine) and its cofactor (tetrahydrobiopterin), as well as an increased K_i (decreased affinity) for its feedback inhibitor (DA). These data suggest a relationship between firing rate and tyrosine hydroxylase activity, at least during periods of increased impulse flow. Similarly, the DA receptor blocker haloperidol has been shown to simultaneously increase firing rate and tyrosine hydroxylase activity (Cooper et al., 1986).

Moreover, Campbell et al. (1986) and Oreland and Engberg (1986) have observed a decrease in firing rate of noradrenergic neurons in the locus ceruleus after an acute high dose of clorgyline (10 mg/kg i.v. or i.p.). Scuvee-Moreau and Dresse (1979) obtained similar results after intraventricular infusion of tranylcypromine (0.05 mg/kg/minute). These data provide evidence for the potential influence of firing rate on tyrosine hydroxylase activity through inhibition of MAO. The effect of inhibition of MAO on firing rate might be expected to be greater in the reserpinized animal in which case both storage and metabolism of newly synthesized DA would be blocked, leading to increased receptor-neurotransmitter interaction as well as increased end product inhibition of

tyrosine hydroxylase by cellular DA. These factors may account for the ability of reserpine to further depress tyrosine hydroxylase activity in clorgyline treated mice (Table 22).

As seen in Table 23, it was not possible to further decrease the rate of reappearance of DA levels after reserpine by providing increased substrate for MAO. Levels of DA and DOPAC were actually somewhat greater in animals receiving both reserpine and chronic L-DOPA (50 mg/kg with 12.5 mg/kg carbidopa i.p. for 7 days followed by a 7-day wash-out period) than in animals receiving reserpine alone. One possible explanation is that reserpine has already induced a maximal effect on the rate of return of DA levels. In support of this idea is the observation that reserpine-induced increases in GSSG, an index of oxidant stress, cannot be further enhanced by L-DOPA administration (200 mg/kg with carbidopa 50 mg/kg i.p., Spina and Cohen, unpublished observation).

In conclusion, it is well known that reserpine causes a sudden and long-lasting depletion of striatal DA levels. Elevations in the acid metabolites DOPAC and HVA are consistent with increased turnover of DA by MAO (Table 16). The rate of return of DA levels after reserpine appears to be somehow affected by reserpine-induced increases in the production of H_2O_2 by MAO in DA neurons (Table 18). This idea is supported by the presence of elevated DA levels in

animals receiving both reserpine and clorgyline after normalization for inhibition of MAO (Table 19). A lack of effect of reserpine on either ^{3}H-DA uptake (Table 20) or ^{3}H-mazindol binding (Table 21), strongly suggests that the effects of reserpine do not include overt toxicity.

Some of the changes seen after reserpine (i.e, low DA levels, and increased DA turnover in intact cells) may bear some relationship to changes seen in mice during normal aging. Finch (1973) and Osterberg et al. (1981) measured a 20-25% decrease in DA levels in the striatum of aged (30 month old) C57BL/6J mice decrease. Similarly, McNeill et al. (1984) reported a marked age-related decrease in DA histofluorescence in the substantia nigra of C57BL/6NNia mice that was associated with an accumulation of lipofuscin pigment and enlarged fluorescent fibers.

It is not clear, however, that decreases in DA levels and histofluorescence in mice represent a loss of neurons. Studies by McNeill and coworkers indicate that while DA levels do decrease (as suggested by decreased fluorescence), the cells themselves are still there. This conclusion was based on the observation that tyrosine hydroxylase staining of nigrostriatal neurons (A9) of aged mice (30 months) was similar to that of young controls (3 months) (McNeill et al., 1984). Similarly, Demarest et al. (1980) observed a 23% decrease in striatal DA levels in aged (24 months) rats as compared to young controls (5 months). However, tyrosine hydroxylase activity in striatum, as measured by the

accumulation of L-DOPA 30 minutes after 100 mg/kg (i.p.) of NSD-1015, was similar in aged and young animals. Moreover, in a recent study, McNeill et al. (1988) counted tyrosine hydroxylase positive cells in the substantia nigra of mice of different ages (3, 6, 10, 20, 25, and 30 months). The number of tyrosine hydroxylase positive cells did not decrease with age with the exception of a small (13%) decrease at 30 months that did not achieve statistical significance. Based on this observation, as well similar results in a comparison of substantia nigra from young and aged human brains, McNeill et al. conclude that reports of decreased DA levels in mice and perhaps humans (as reviewed by Morgan and Finch (1988), cannot be explained by a loss of nigrostriatal neurons.

The current study indicates that a decrease in tyrosine hydroxylase may explain a portion of the persistent decrease in DA seen after reserpinization in mice. Overt destruction of nerve terminals and diminished vesicular storage capacity appear to be excluded. One factor that was not studied was the regulation of DA release by autoreceptors. The latter area deserves closer scrutiny because presynaptic regulation can affect DA turnover, which remains persistently increased at 3 weeks after reserpinization (Table 17). These studies indicate that the potential relationships among (1) decreased DA seen in aging rodent brain, (2) decreased DA accompanied by increased DA

turnover seen in aging human brain, and (3) persistently decreased DA accompanied by increased DA turnover seen at 3 weeks after reserpine, are worthy of further detailed investigation.

References

Aghajanian G. K., Cedarbaum J. M., and Wang R. Y. (1977) Evidence for norepinephrine-mediated collateral inhibition of locus ceruleus neurons. Brain. Res. 136, 570-577.

Algeri S., Achilli G., Calderini G., Perego C., Ponzio F., and Toffano G. (1987) Age-related changes in metabolic responses to chronic monoamine depletion in central dopaminergic and serotonergic systems of rats treated with reserpine. Neurobiol. of Aging. 8, 61-66.

Anden N. -E, Magnusson T., and Waldeck B. (1964) Correlation between noradrenaline uptake and adrenergic nerve function after reserpine treatment. Life Sci. 3, 19-25.

Beal M., Kowall N. W., Ellison D. W., Mazurek M. F., Schwartz K. J., and Martin J. B. (1986) Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. Nature. 321, 168-171.

Berkowitz B.A., Spector S., Brossi A., Focella A., and Teitel S. (1970) Preparation and biological properties of (-)- and (+)-6-hydroxydopa. Experientia. 29, 982-983.

Berge O-G, Fasmer O. B., Tveiten L., and Hole K. (1985) Selective neurotoxic lesions of descending serotonergic and noradrenergic pathways in the rat. J. Neurochem. 44, 1156-1161.

Bernheimer H., Birkmayer W., Hornykiewicz O., Jellinger K., and Seitelberger F. (1973) Brain dopamine and the syndromes of Parkinson and Huntington. J. Neurol. Sci. 20, 415-455.

Bjorklund A. and Lindvall A. (1975) Dopamine in dendrites of substantia nigra neurons: suggestions for a role in dendritic terminals. Brain Res. 83, 531-537.

Boyland E. and Chasseaud L. F. (1967) Enzyme-catalyzed conjugations of glutathione with unsaturated compounds. Biochem. J. 104, 95-102.

Boyland E. and Chasseaud L. F. (1970) The effect of some carbonyl compounds on rat liver glutathione levels. Biochem. Pharmacol. 19, 1526-1528.

Braestrup C. and Scheel-Kruger J. (1976) Methylphenidate-like effects of the new antidepressant drug nomifensine (Hoe 984). Eur. J. Pharmacol. 38, 305-312.

Breese G. R. and Traylor T.D. (1971) Depletion of brain noradrenaline and dopamine by 6-hydroxydopamine. Br. J. Pharmacol. 42, 88-99.

Brigelius R. (1985) Mixed disulfides: Biological functions and increase in oxidative stress, in Oxidative Stress (Sies H. ed.) pp. 243-272, Academic Press, London.

Buu N. T. and Angers M (1987) Effects of different monoamine oxidase inhibitors on the metabolism of L-dopa in the rat brain. Biochem. Pharmacol. 36, 1731-1735.

Calvin H., Medovsky C., and Worgul B. V. (1986) Near-total glutathione depletion and age specific cataracts induced by buthionine sulfoximine in mice. Science 233, 553-555.

Campbell I. C., Gallager D. W., Hamburg M. A., Tallman J. F., and Murphy D. L. (1985) Electrophysiological and receptor studies in rat brain: Effects of clorgyline. Eur. J. of Pharmacol. 111, 355-364.

Carlsson A., Rosengren E., Bertler A., and Nilsson J. (1957) Effect of reserpine on the metabolism of catecholamines in Psychotropic Drugs (Garattini S. and Ghetti V., eds.) pp.363-372, Elsevier Publ. Co., Amsterdam.

Carlsson A., Hillarp N. -A. and Waldeck B. (1963) Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. Acta Physiol. Scand. 59, Suppl. 215, 1-38.

Cedarbaum J. M. and Aghajanian G. K. (1977) Catecholamine receptors on locus ceruleus neurons: pharmacological characterization. Eur. J. Pharmacol. 44, 375-385.

Clarke D.E., Smookler H.H., Hadinata J., Chi C., Barry III H. (1972) Acute effects of 6-hydroxydopa an its interaction with dopa on brain amine levels. Life Sci. 11, 97-102.

Cohen G. (1983) The pathobiology of Parkinson's disease: Biochemical aspects of dopamine neuron senescence. J. Neural. Trans., Suppl. 19, 89-103.

Cohen G. (1986) Monoamine oxidase, hydrogen peroxide, and Parkinson's disease. Adv. Neurol. 45, 119-125.

Cohen G. and Spina M. B. (1989) Deprenyl suppresses the oxidant stress associated with increased dopamine turnover. Ann. Neurol. In Press.

Cooper J., Bloom F. E., and Roth R. H. (1986) The Biochemical Basis of Neuropharmacology, Oxford University Press, New York.

Cooper A. J., Pulsinelli W. A., and Duffy T. E. (1980) Glutathione and ascorbate during ischemia and postischemic

reperfusion in rat brain. J. Neurochem. 35, 1242-1245.

Cornwell-Jones C. A. and Bollers H. R. (1983) Neonatal 6-hydroxydopa alters conspecific odor investigation by male rats. Brain Res. 268, 291-294.

Corrodi H. and Fuxe K. (1969) Decreased turnover in central 5-HT nerve terminals induced by antidepressant drugs of the imipramine type. Eur. J. Pharmacol. 7, 56-66.

Corrodi H., Clark W.G., and Masuoki D.I. (1971) The synthesis and effects of DL-6-hydroxydopa in 6-Hydroxydopamine and Catecholamine Neurons (Malforms T. and Thoenen H., eds.) pp. 187-192, North-Holland Publishers, Amsterdam.

Dahlstrom A., Fuxe K., and Hillarp N-A. (1965) Site of action of reserpine. Acta pharmacol. et toxicol. 22, 277-292.

Dahlstrom A. and Haggendal J. (1966) Recovery of noradrenaline levels after reserpine compared with the life-span of amine storage granules in rat and rabbit. J. Pharm. Pharmacol. 18, 750-752.

Davies P. and Verth A. H. (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. Lancet. 2, 1403.

Demarest K. T., Riegle G. D., and Moore K. E. (1980) Characteristics of dopaminergic neurons in the aged male rat. Neuroendocrinology. 31, 222-227.

Deupree J. D. and Hitchcock J. J. (1988) Effects of the sulfhydryl reagent n-ethylmaleimide on reserpine binding to the catecholamine transporter in chromaffin granule vesicles. Cell. Mol. Neurobiol. 8, 217-224.

Dexter D., Carter C., Agid F., Agid Y., Lees A. J., Jenner P., and Marsden C. D. (1986) Lipid peroxidation as a cause of nigral cell death in Parkinson's disease. Lancet ii, 639-640.

Dexter D. T., Wells F. R., Agid F., Agid Y., Lees A. J., Jenner P., and Marsden C. D. (1987) Increased nigral iron content in postmortem Parkinsonian brain. Lancet.*1219-1220.

Dexter D. T., Wells F. R., Lees A. J., Agid F., Agid Y., Jenner P. and Marsden C. D. (1989a) Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease (1989) J. Neurochem. 53, 1830-1836.

Dexter D. T., Carter C. J., Wells F. R., Javoy-Agid F., Agid

Y., Lees A., Jenner P., and Marsden C. D. (1989b) Basal lipid peroxidation in Parkinson's disease. J. Neurochem. 52, 381-389.

Doucet G., Descarries L., and Garcia S. (1986) Quantification of the dopamine innervation of the neostriatum. Neuroscience. 19, 427-445.

Ehringer H. and Hornykiewicz O. (1960) Verteilung von Noradrenalin und Dopamin (3-Hydroxytryptamin) im Gehirn des Menschen und ihr Verhalten bei Erkrankungen des extrapyramidalen Systems. Klin. Wochenschr. 38, 1236-1239

Evans J. M. and Cohen G. (1989a) Studies on the formation of 6-hydroxydopamine in mouse brain after administration of 2,4,5-trihydroxyphenylalanine (6-hydroxydopa). J. Neurochem. 52, 1461-1467.

Evans J. M. and Cohen G. (1989b) Can trace amounts of neurotoxins destroy dopamine neurons? Neurochem. Int. In Press.

Faucon Biguet N., Buda M., Lamouroux A., Samolyk D., and Mallet J. (1986) Time course of the changes of TH mRNA in rat brain after i single injection of reserpine. EMBO J. 5, 287-291.

Finch C. E. (1973) Catecholamine metabolism in the brains of ageing male mice. Brain Res. 52, 261-276.

Gilbert H. F. (1982) Biological disulfides: The third messenger? Modulation of phosphofructokinase activity by thiol/disulfide exchange. J. Biol. Chem. 257, 12086-12091.

Glowinski J. and Iversen L. (1966) Regional studies of catecholamines in rat brain I. J. Neurochem. 13, 655-669.

Glowinski J., Iversen L. L., and Axelrod J. (1966) Storage and synthesis of norepinephrine in the reserpine treated rat brain. J. Pharm. Exp. Ther. 151, 385-399.

Graham D. G., Tiffany S. M., Bell Jr. W. R., and Gutknecht W. F. (1978) Autoxidation versus covalent binding of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. Mol. Pharmacol. 14, 644-653.

Grima B., Lamouroux A., Blanot F., Faucon Biguet N., and Mallet J. (1985) Complete coding sequence of rat tyrosine hydroxylase mRNA. Proc. Nat. Acad. Sci. 82, 617-621.

Haggendal J. and Dahlstrom A. (1971) The recovery of noradrenaline in adrenergic nerve terminals of the rat after

reserpine treatment. J. Pharm. Pharmacol. 23, 81-89.

Halliwell B. and Gutteridge J. M. C. (1986) Iron and free radical reactions: two aspects of antioxidant protection. Trends in Biochem. Sci. 11, 372-375.

Haskins J. T., Moyer J. A., Muth E. A., and Sigg E. B. (1985) DMI, WY-45,030, WY-45,881 and ciramadol inhibit locus ceruleus neuronal activity. Eur. J. Pharmacol. 115, 139-146.

Hefti F., Melamed E., and Wurtman R. J. (1980) Partial lesions of the dopaminergic nigrostriatal system in rat brain: Biochemical characterization. Brain Res. 195, 123-137.

Hefti F., Melamed E., and Wurtman R. J. (1981) The site of dopamine formation in rat striatum after L-dopa administration. J. Pharmacol. Exp. Ther. 217, 189-197.

Heikkila R. and Cohen G. (1972) Further studies on the generation of hydrogen peroxide by 6-hydroxydopamine: potentiation by ascorbic acid. Mol. Pharmacol. 8, 241-248.

Heikkila R. E., Mytilineou C., Cote L. and Cohen G. (1973) Evidence for degeneration of sympathetic nerve terminals caused by the ortho- and para-quinones of 6-hydroxydopamine. J. Neurochem. 20, 1345-1350.

Hornykiewicz O. (1979) Compensatory biochemical changes at the striatal dopamine synapse in Parkinson's disease-Limitations of L-dopa therapy. Adv. Neurol. 24, 275-281.

Hornykiewicz O. (1983) Dopamine changes in aging human brain functional considerations in Aging Brain and Ergot Alkaloids (Agnoli A., ed.) pp. 9-14, Raven Press, New York.

Hornykiewicz O. (1988) Plenary lecture: 2nd International Conference on MPTP (Degeneration and Regeneration Processes in CNS), Cagliari, Sardinia, Italy.

Iversen L.L. and Uretsky N. J. (1971) Biochemical effects of 6-hydroxydopamine on catecholamine-containing neurones in the rat central nervous system in 6-Hydroxydopamine and Catecholamine Neurons (Malforms T. and Thoenen H., eds.) pp. 171-186, North-Holland Publishers, Amsterdam.

Jacobowitz D. M. (1973) Effects of 6-hydroxydopa. in Frontiers in Catecholamine Research (Usdin E. and Synder S. H., eds.) pp. 729-739.

Jacobowitz D. and Kostrzewa R. (1971) Selective action of 6-hydroxydopa on noradrenergic nerve terminals: Mapping of preterminal axons of the brain. Life Sci. 10, 1329-1342.

Javitch J. A., Blaustein R. O., and Snyder S. H. (1984) [^{3}H] Mazindol binding associated with neuronal dopamine and norepinephrine uptake sites. Mol. Pharmacol. 26, 35-44.

Javoy F., Agid Y., Bouvet D., and Glowinski J. (1972) Feedback control of dopamine synthesis in dopaminergic terminals in the striatum. J. Pharmacol. Exp. Ther. 182, 454-463.

Johnston J. P. (1968) Some observations on a new inhibitor of monoamine oxidase in brain tissue. Biochem. Pharm. 17, 1285-1297.

Jonsson G. and Sachs C. (1970) Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurons. Eur. J. Pharmac. 9, 141-155.

Jonsson G. and Sachs C. (1971) Uptake and accumulation of ^{3}H-6-hydroxydopamine in adrenergic nerves. Eur. J. Pharmacol. 16, 55-62.

Kantak K. M., Hegstrand L. R., and Eichelman B. (1981) Facilitation of Shock induced fighting following intraventricular 5,7-dihydroxytryptamine and 6-hydroxydopa. Psychopharmacol. 74, 157-160.

Kostrzewa R. M. and Jacobowitz D. M. (1972) The effect of 6-hydroxydopa on peripheral adrenergic neurons. J. Pharmacol. Exp. Ther. 183, 284-297.

Kostrzewa R. M. and Jacobowitz D. M. (1973) Acute effects of 6-hydroxydopa on central monoaminergic neurons. Eur. J. Pharmacol. 21, 70-80.

Kostrzewa R. M. and Jacobowitz D. M. (1974) Pharmacological actions of 6-hydroxydopamine. Pharmacol. Revs. 26, 199-288.

Labutat R., Buda M., Berod A. (1988) Long-term changes in rat brain tyrosine hydroxylase following reserpine treatment: A quantitative immunochemical analysis. J. Neurochem. 50, 1375-1380.

Liang Y. -O., Plotsky P. M., and Adams R. N. (1977) Isolation and identification of an in vivo reaction product of 6-hydroxydopamine. J. Amer. Chem. Soc. 20, 581-583.

Maker H. S., Weiss C., Silides D. J. and Cohen G. (1981) Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates. J. Neurochem. 36, 589-593.

Mann D. M. A. (1984) Dopamine neurons of the vertebrate brain: Some aspects of anatomy and pathology in The neurobiology of dopamine systems (Winlow W. and Markstein R., eds.) pp. 87-103, Manchester University Press, Manchester.

Mann D. M. A and Yates P. O. (1983) Possible roles of neuromelanin in the pathogenesis of Parkinson's disease. Mechn. Aging Development 21, 193-203.

Matsumura M. and Matthews B. W. (1989) Control of enzyme activity by an engineered disulfide bond. Science. 243, 792-794.

McClean J. H., Glasser R. S., Kostrzewa R. M. and May J. G. (1980) Effects of neonatal 6-hydroxydopa on behavior in female rats. Pharmacol. Biochem. and Behav. 13, 863-868.

McGeer P. L., McGeer E. G., and Suzuki J. S. (1977) Aging and extrapyramidal function. Arch. Neurol. 34, 33-35.

McNeill T. H., Koek L. L., and Haycock J. W. (1984) Age-correlated changes in dopaminergic perikarya of the C57BL/6NNia mouse. Mech. of Aging and Dev. 24, 293-307.

McNeill T. H., Koek L., and Haber S.N. (1988) Differential effects of neurotransmitter cell loss of the substantia nigra and striatum in mouse and human brain. Soc. for Neurosci. Abstracts. 14, 1292.

Melamed E., Hefti F., Pettibone D. J., Liebman J., and Wurtman R. J. (1981) Aromatic L-amino acid decarboxylase in the rat corpus striatum: Implications for actions of L-dopa in parkinsonism. Neurology 31, 651-655.

Morgan D. G. and Finch C. F. (1988) Dopaminergic changes in the basal ganglia: A generalized phenomenon of aging in mammals. Ann. N.Y. Acad. Sci. 515, 145-160.

Murrin L. C., Morgenroth V. H. III, and Roth R. H. (1976) Dopaminergic neurons: Effects of electrical stimulation on tyrosine hydroxylase. Mol. Pharmacol. 12, 1070-1081.

Nieoullon A., Cheramy A., and Glowinski J. (1977) Release of dopamine in vivo from cat substantia nigra. Nature, 266, 375-377.

Nissbrandt H. and Carlsson A. (1987) Turnover of dopamine and dopamine metabolites in rat brain: Comparison between striatum and substantia nigra. J. Neurochem. 49, 959-967.

Nissbrandt H., Sundstrom E., Jonsson G., Hjorth S. and Carlsson A. (1989) Synthesis and release of dopamine in rat

brain: Comparison between substantia nigra pars compacta, pars recticulata, and striatum. J. Neurochem. 52, 1170-1182.

Nyback H. V., Walters J. R., Aghajanian G. K., and Roth R. H. (1975) Tricyclic antidepressants: Effects on the firing rate of brain noradrenergic neurons. Eur. J. Pharmacol. 32, 302-312.

Oberhauser E., Weinstein V., and Asjeno A. (1970) Iron concentration in the basal ganglia and cerebral cortex obtained by overloading rabbits and monkeys with iron dextran. J. Neuro. Surg. 32, 677-679.

Offerman M. K., McKay M. J., Marsh M. W., Bond J. S. (1984) Glutathione disulfide inactivates, destabilizes, and enhances proteolytic susceptibility of fructose-1,6-bisphosphate aldolase. J. Biol. Chem. 259, 8886-8891.

Olpe H. R., Jones, R. S. G., Steinmann M. W. (1983) The locus ceruleus: actions of psychotropic drugs. Experientia. 39, 242-249.

Ong H. H., Creveling C. R., and Daly J. W. (1969) The synthesis of 2,4,5-trihydroxyphenylalanine (6-hydroxydopa). A centrally active norepinephrine- depleting agent. J. Med. Chem. 12, 458-461.

Oreland L. and Engberg G. (1986) Relation between brain monoamine oxidase (MAO) activity and the firing rate of locus ceruleus neurons. Nauyn-Schmiedeberg's Arch. Pharmacol. 333, 235-239.

Oshino N. and Chance B. (1977) Properties of glutathione release observed during reduction of organic hydroperoxide, demethylation of aminopyrine and oxidation of some substances in perfused rat liver, and their implications for the physiological function of catalase. Biochem. J. 162, 509-525.

Osterburg H. H., Donahue H. G., Severson J. A. and Finch C. E. (1981) Catecholamine levels and turnover during aging in brain regions of male C57BL/6J mice. Brain Res. 224, 337-352.

Pileblad E., Magnusson T, and Fornstedt B. (1989) Reduction of brain glutathione by L-buthionine sulfoximine potentiates the dopamine-depleting action of 6-hydroxydopamine in rat striatum. J. Neurochem. 52, 978-990.

Physicians' Desk Reference (1986), 40th Edition, p. 1210, Medical Economics Co., Inc, Oradell, New Jersey.

Plummer J. L., Smith B. R., Sies H., and Bend J. R. (1981)

Chemical depletion of glutathione in Vivo, in Methods in Enzymology (Jakoby W. B. ed.) pp. 50-59, Academic Press, New York.

Ponzio F., Achilli G., Calderini, G., Ferretti P., Perego, C., Toffano G., and Algeri S. (1984) Depletion and recovery of neuronal monoamine storage in rats of different ages treated with reserpine. Neurobiol. of Aging. 5, 101-104.

Quinn G. P., Shore P. A., and Brodie B. B. (1959) Biochemical and pharmacological studies of RO1-9569 (tetrabenazine), a non-indole tranquilizing agent with reserpine-like effects. J. Pharmacol. Exp. Ther. 127, 103-109.

Racagni G., Mocchetti I., De Angelis L., Baraldi M., and Cuomo E. (1982a) In vivo effects of typical and atypical antidepressants on brain noradrenergic system: Behavioural Correlations. Adv. Biochem. Psychopharmacol. 31, 185-197.

Racagni G., Mochetti I., Renna G., and Cuomo V. (1982b) In vivo studies on central noradrenergic synaptic mechanisms after acute and chronic antidepressant drug treatment: biochemical and behavioral comparison. J. Pharmacol. Exp. Ther. 223, 227-234.

Reinhard J. F. Jr., Daniels A. J. and Viveros O. H. (1988) Potentiation by reserpine and tetrabenazine of brain catecholamine depletions by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in the mouse; evidence for subcellular sequestration as basis for cellular resistance to the toxicant. Neurosci. Letts. 90, 349-353.

Reis D. J., Joh T. H., and Ross R. A. (1975) Effects of reserpine on activities and amounts of tyrosine hydroxylase and dopamine beta-hydroxylase in catecholamine neuronal systems in rat brain. J. Pharmacol. Exp. Ther. 193, 775-784.

Richardson J. S. and Jacobowitz D. M. (1973) Depletion of brain norepinephrine by intraventricular injection of 6-hydroxydopa: a biochemical, histochemical, and behavioral study in rats. Brain Res. 58, 117-133.

Rollema H., De Vries J. B., Westerink B. H. C., Van Putten F. M. S., and Horn A. S. (1986) Failure to detect 6-hydroxydopamine in rat striatum after the dopamine releasing drugs dexamphetamine, methylamphetamine, and MPTP. Eur. J. Pharmacol. 132, 65-69.

Sachs C. and Jonsson G. (1972a) Degeneration of central and peripheral noradrenaline neurons produced by 6-hydroxy-dopa. J. Neurochem. 19, 1561-1575.

Sachs C. and Jonsson G. (1972b) Selective 6-hydroxy-DOPA induced degeneration of central and peripheral noradrenaline neurons. Brain Res. 40, 563-568.

Schoepp D. D. and Azzaro A. J. (1981) Alteration of dopamine synthesis in rat striatum subsequent to selective Type A monoamine oxidase inhibition. J. Neurochem. 37, 527-530.

Schubert J., Nyback H., and Sedvall G. (1970) Effect of antidepressant drugs on accumulation and disappearance of monoamines formed in vivo from labelled precursors in mouse brain. J. Pharm. Pharmacol. 22, 136-139

Scuvee-Moreau J. J. and Dresse A. E. (1979) Effect of antidepressant drugs on the spontaneous firing rate of locus ceruleus and dorsal raphe neurons of the rat. Eur. J. Pharmacol. 57, 219-225.

Scuvee-Moreau J. and Dresse A. (1982) Effect of trazodone on the firing rate of central monoaminergic neurons. Comparison with various antidepressants. Arch. Int. Pharmacodyn. 260, 299-301.

Seiden L. S. and Vosmer G. (1984) Formation of 6-hydroxydopamine in the caudate nucleus of rat brain after a single large dose of methylamphetamine. Pharmacol. Biochem. Behav. 21, 29-31.

Senjo M. and Ishibashi T. (1985) Specific localization of glutathione S-transferases in astrocytes and ependymal cells of rat brain: Immunocytochemical demonstration. Biomed. Res. 6, 433-436.

Senoh S., Creveling C. R., Udenfriend S. and Witkop B. (1959) Chemical, enzymatic and metabolic studies on the mechanism of oxidation of dopamine. J. Am. Chem. Soc. 81, 6236-6240.

Sershen H., Mason, M. F., Reith M. E. A., Hashim A., and Lajtha (1986) Effect of nicotine and amphetamine on the neurotoxicity of N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice. Neuropharmacol. 25, 1231-1234.

Slivka A. and Cohen G. (1985) Hydroxyl radical attack on dopamine. J. Biol. Chem. 260, 15466-15471.

Slivka A., Mytilineou C., and Cohen G. (1987) Histochemical evaluation of glutathione in brain. Brain Res. 409, 275-284.

Slivka A, Spina M. B., Calvin H. I., and Cohen G. (1988) Depletion of brain glutathione in preweanling mice by L-Butathione Sulfoximine. J. Neurochem. 50, 1391-1393.

Sofic E., Riederer P., Heinsen H., Beckmann H., Reynolds G. P., Hebenstreit G., and Youdim M. B. H. (1988) Increased iron (III) and total iron content in post mortem substantia nigra of Parkinsonian brain. J. Neural. Trans. 74, 199-205.

Spina M. B. and Cohen G. (1989) Dopamine turnover and glutathione oxidation: Implications for Parkinson's disease. Proc. Natl. Acad. Sci. 86, 1398-1400.

Studer A. and Schultz W. (1987) The catecholamine uptake inhibitor nomifensine depresses impulse activity of dopamine neurons in the mouse substantia nigra. Neurosci. Lett. 80, 207-212.

Tassin J. P., Velley L., Stinus L., Blanc G., Glowinski J., and Thierry A. M. (1975) Development of cortical and nigro-neostriatal dopaminergic systems after destruction of central noradrenergic neurones in foetal or neonatal rats. Brain Res. 83, 93-106.

Tietze K. F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Analyt. Biochem. 7, 502-522.

Triggs W. J. and Willmore L. J. (1984) In vivo lipid peroxidation in rat brain following intracortical Fe^{2+} injection. J. Neurochem. 42, 976-980.

VonVoigtlander P. F. and Losey E. G. (1978) 6-Hydroxydopa depletes both brain epinephrine and norepinephrine: interactions with antidepressants. Life Sci. 23, 147-150.

Willmore L. J., Hiramatsu H. K., and Mori A. (1983) Formation of superoxide radicals after $FeCl_3$ injection into rat isocortex. Brain Res. 277, 393-396.

Willmore L. J. and Rubin J. J. (1984) The effect of tocopherol and dimethyl sulfoxide on focal edema and lipid peroxidation induced by isocortical injection of ferrous chloride. Brain Res. 296, 389-392.

Willmore L. J., Triggs W. J., and Gray J. D. (1986) The role of iron-induced hippocampal peroxidation in acute epileptogenesis. Brain Res. 382, 422-426.

Wood P. L., Kim H. S., and Altar A. (1987) In vivo assessment of dopamine and norepinephrine release in rat neocortex: Gas chromatography-mass spectrometry measurement of 3-methoxy tyramine and normetanephrine. J. Neurochem. 48, 574-579.